

AD _____

GRANT NUMBER DAMD17-96-1-6324

TITLE: 99: A Novel Myc-Interacting Protein with Features of a
Breast Tumor Suppressor Gene Product

PRINCIPAL INVESTIGATOR: George Prendergast, Ph.D.

CONTRACTING ORGANIZATION: The Wistar Institute
Philadelphia, PA 19104-4268

REPORT DATE: October 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

19980205 109

DEC 30 1997
QUALITY INSPECTED

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE October 1997	3. REPORT TYPE AND DATES COVERED Annual (15 Sep 96 - 14 Sep 97)	
4. TITLE AND SUBTITLE 99: A Novel Myc-Interacting Protein with Features of a Tumor Suppressor Gene Product			5. FUNDING NUMBERS DAMD17-96-1-6324	
6. AUTHOR(S) George Prendergast, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Wistar Institute Philadelphia, PA 19104-4268			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick, Frederick, Maryland 21702-5012			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200) Bin1 is a novel tumor suppressor-like molecule we identified through its ability to interact with and inhibit the oncogenic activity of the Myc oncoprotein, which is widely deregulated in breast cancer. Last year, we proposed to test the hypothesis that Bin1 was a breast tumor suppressor molecule. In year one of the project, we completed characterization of Bin1 antibodies useful for immunohistochemistry and the cloning and characterization of the entire ≥ 53 kb human Bin1 gene and 5' flanking region. Using these reagents, we demonstrated that (1) Bin1 is expressed in normal breast epithelial cells but very frequently missing in malignant breast tumors, and that (2) the Bin1 gene was altered as the potential basis for lack of expression in 3/6 tumor cell lines examined. In preparation to assess the functional of genetic abnormalities whose identification is ongoing, we developed mechanism-based assays for malignant growth inhibition by Bin1. Finally, in preparation for biological analyses, we developed a recombinant Bin1 adenovirus and an MCF7 inducible gene system.				
14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 83	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

_____ Where copyrighted material is quoted, permission has been obtained to use such material.

_____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

_____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

_____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

_____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

_____ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

_____ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

_____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.



PI - Signature

10-3-97

Date

Table of Contents

	Page
Foreword.....	3
Table of Contents.....	4
Introduction.....	5
Body.....	5
Conclusions.....	9
References.....	10
Appendices.....	11

Introduction

An important goal in breast cancer research is to identify better prognostic tools to predict the course and relapse of malignant carcinoma, and to uncover and develop foundations for the development of novel modalities to treat advanced, intractable disease. Malignant breast carcinomas frequently contain deregulated Myc (Shiu *et al.* 1993). Notably, deregulation is most frequently seen in advanced tumors (due to genetic or epigenetic causes) and, where it occurs, signals poor prognosis (Berns *et al.* 1992; Borg *et al.* 1992; Hehir *et al.* 1993; Watson *et al.* 1993). Supporting the importance of the Myc system in breast cancer, the Myc-regulated genes plasminogen activator inhibitor-1 (Prendergast *et al.* 1989; Prendergast *et al.* 1990) and ornithine decarboxylase (Bello-Fernandez *et al.* 1993) are also indicators of poor prognosis (Reilly *et al.* 1992; Sumiyoshi *et al.* 1992; Manni *et al.* 1995). The ability of deregulated Myc to drive apoptosis may provide an Achilles' heel in such cells. Indeed, the ability of the anti-breast cancer drug tamoxifen appears to use Myc-mediated death mechanisms to exert its activity (Kang *et al.* 1996). Therefore, unraveling Myc death mechanisms represent one direction to address a major clinical need.

As part of an effort to learn how deregulated Myc kills cells, we identified the Myc-binding protein Bin1 (formerly called 99). In the background to our proposal we summarized the circumstantial evidence pointing to a role for Bin1 as a breast tumor suppressor gene. In the proposed project, we specifically aimed to:

1. Identify gene mutations and loss of expression in tumor cell lines and primary tumors.
Tasks 1-4 planned for start in year 1 of the grant were all initiated. Those scheduled for completion were indeed finished but the scope has been broadened to increase the significance of what were previously planned to be pilot findings.
2. Ectopically express Bin1 in human tumor and model rodent cell systems and assay its effects on malignant cell growth, cell cycle progression, and apoptotic index.
None of the tasks planned were scheduled for initiation in this period.
3. Mutate Bin1 and assay the mutants for growth inhibitory and/or apoptosis activity.
None of the tasks planned were scheduled for initiation in this period.

Bin1 structure in normal and malignant breast cells

Aim 1. Identify mutations and loss of expression in tumor cell lines and primary tumors

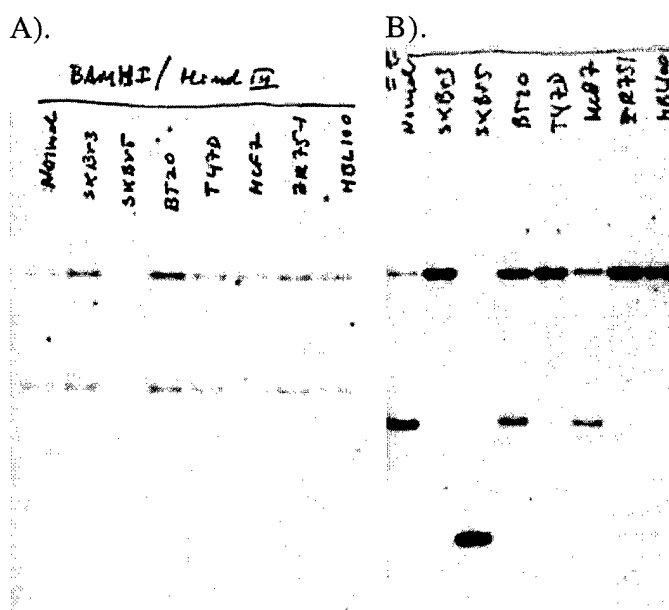
Task 1: Northern and Southern analysis (months 1-12)

Northern analysis. Tumor cell lines, primary tumors, and normal tissues were each analyzed as planned. We observed that 5/6 breast tumor cell lines had greatly reduced or undetectable levels of Bin1 message (HBL100 cells were used as a "normal" cell control in this assay since we have found they express levels of Bin1 similar to those found in normal breast tissue). Confirming that this was not a cell line artifact, 3/6 primary tumors also showed undetectable message levels (the RNAs from four additional primary tumors were too degraded during the preparation to give interpretable results; this set of 10 tumors constituted the entire analysis proposed in the pilot study in year 1 of the project). Normal tissues were also examined for Bin1 message. We found that all

normal tissues expressed Bin1 at a similar basal level. These results were published as part of our initial Bin1 publication (see Appendices for reprint) (Sakamuro *et al.* 1996). One of the conclusions of this study was that loss of Bin1 message was a frequent occurrence in breast cancer cells.

Southern Analysis. The frequent loss of expression in breast cancer cells prompted us to hypothesize that the Bin1 gene may be deleted. To examine this possibility, we performed Southern analysis on tumor cell line DNA. Southern blots probed with a Bin1 cDNA indicated that in one non-expressing cell line, SK-BR-5, the gene was almost wholly deleted, and in a second, SK-BR-3, the locus was abnormal (see Figure 1). We extended this analysis to the 5' end of the gene after its cloning and characterization was completed (Wechsler-Reya *et al.* 1997). This part of the gene, which includes only exon 1 in addition to the 5' flanking region, is ~50 kb upstream of the main body of the gene which is seen by the cDNA probe. Consistent with the other data, SK-BR-5 also showed deletion in the 5' end. Other differences in the 5' end appeared to reflect a restriction length polymorphism (Figure 1 and data not shown). In conclusion, 1/6 breast tumor cell lines exhibited Bin1 deletion. However, this begged the question why Bin1 expression was lost in the other 4/5 tumors showing lack of message.

Figure 1. Bin1 gene status in breast cancer cell lines. Genomic DNAs were probed with Bin1 cDNA (A.) or a genomic Xba I fragment encompassing the 5' flanking region and exon 1 of the gene. SK-BR-5 exhibited extensive deletion at this locus; SK-BR-3 had abnormal organization.



This analysis is currently being extended to a set of 16 tumor + matched normal breast tumors that we have accumulated. In addition to Southern analysis, we will also look for loss-of-heterozygosity (LOH) in pairs of normal/tumor DNA, using a highly polymorphic microsatellite marker we have identified in intron 5 of the Bin1 gene (Wechsler-Reya *et al.* 1997).

Since gross gene deletion is not seen, but there is a frequent lack of message, we are extending the Southern analysis to examine the hypothesis that aberrant gene methylation leads to epigenetic suppression of gene expression. This test will employ genomic DNAs digested by methylation-sensitive or -insensitive restriction enzymes, blotted, and hybridized to 5' end probes. The expectation is that where RNA is expressed (e.g. HBL100) we will see fewer signs of

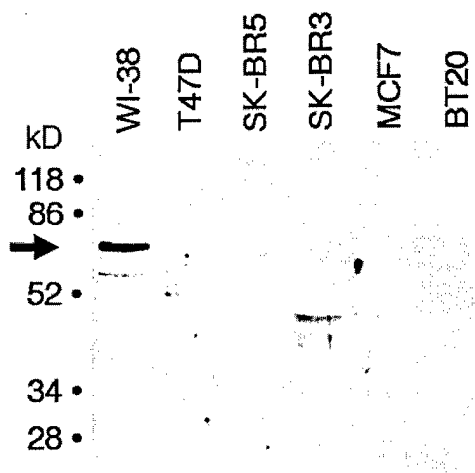
methylation than in tumor cell lines (e.g. ZR-75-1) where Bin1 message is absent but no signs of gene abnormality are detected.

Task 2. Immunoprecipitation analysis (months 1-12)

To confirm loss of expression in tumor cells, we have performed Western blotting of tumor cell lines and immunohistochemistry of primary tumors (each a more desirable measurement of steady-state protein levels than immunoprecipitation [IP]).

Western analysis confirmed loss of expression in all lines that showed no Bin1 message. Furthermore, it extended loss of expression to 6/6 tumor cell lines by revealing lack of protein in BT-20, that had exhibited levels of message similar to HBL100, and a truncated protein in SK-BR-3, which had showed gene abnormality by Southern (see Figure 2). These findings argue that the messages in BT-20 and SK-BR-3 are mutated, although we have yet to confirm this. To extend and confirm these findings in primary tumors, we performed immunohistochemistry on 3 primary tumors and 3 normal tissues. We observed that each normal sample showed the expected pattern of nuclear staining, confirming expression in normal breast ductal epithelial cells. In stark contrast, none of the tumor sections exhibited staining. These results were reported in our initial report on Bin1 (see Appendices for reprint) (Sakamuro *et al.* 1996).

Figure 2. Bin1 Western analysis in breast tumor cell lines. WI-38 normal diploid fibroblasts serves as a positive for expression. The results show that even tumor lines that express Bin1 message lack normal Bin1 protein (SK-BR-3 shows a mutated truncated protein where as BT-20 shows none at all).



We are currently working to expand the immunohistochemical analysis using a larger sample size of breast tumor sections.

Task 3. Genomic PCR (months 6-36)

Limited LOH analysis of tumor + matched normal samples of genomic DNA using a polymorphic microsatellite in the Bin1 gene have not revealed positive results to date, suggesting that gross deletion of the gene is not common. However, we have not yet examined a sufficient number of DNA pairs to make any definitive conclusions.

Task 4: Hybrid mismatch analysis (months 1-48)

We have not yet initiated this analysis. Instead, in its place we have begun an RT/PCR and DNA sequencing project to directly identify alterations in tumor messages (where present). We will also perform this analysis on message expressed in the 3/6 primary tumors initially identified and any that we find in the additional 16 tumors currently being examined.

Aim 2. Ectopically express Bin1 in human tumor and model rodent cell systems and assay its effects on malignant cell growth, cell cycle progression, and apoptotic index.Tasks 5,6,7. Biological assays.

Although these tasks were not scheduled for initiation in this grant period, we have moved ahead and begun them. Preliminary results showing that Bin1 can suppress the oncogenic activity of not only Myc, but also the adenovirus E1A, HPV E7, and mutant p53 genes has been obtained. This activity is selective insofar as Bin1 did not affect the oncogenic activity of the SV40 large T antigen. This line of work is informative because it suggests that Bin1 can inhibit malignant cell growth through both Myc-dependent and Myc-independent mechanisms, broadening its potential application in breast cancer. In addition, this line of work suggests that Bin1 may interact with the Rb/E2F system in the cell (which is targeted by the E1A and E7 proteins in transformed cells) and with the p53 system, which is frequently altered in many types of carcinoma, including breast cancer.

In an effort to obtain appropriate vector and cell line systems, we have developed a recombinant Bin1 adenovirus and are in the process of characterizing a set of MCF7 cell lines containing inducible Bin1 genes (based on the sheep metallothionein promoter, which we have found to be sufficiently non-leaky for use). The adenoviral vector has been confirmed for Bin1 protein expression; we are currently sequencing it to confirm an intact wild-type coding region.

Aim 3. Mutate Bin1 and assay the mutants for growth inhibitory and/or apoptotic activity.Tasks 8,9: Generation and of Bin1 mutants and analysis of their biological activities.

Although these tasks were not scheduled for initiation in this grant period, we have moved ahead and begun them. A set of deletion mutants in Bin1 has been developed and assayed for inhibitory activity against the Myc, E1A, mutant p53, and E7 oncogenes in a model rodent fibroblast transformation system. This work has revealed where the important functional domains of Bin1 lie, a first step in assessing the prospects for development of therapeutic approaches.

This work was important to start earlier than originally planned because we realized that as we defined mutations in the Bin1 gene in breast cancer cells, we would need mechanism-dependent assays to determine whether they represented polymorphisms or "silent" changes or true loss-of-function mutations. With these assays in place, we are now in a better position to confirm genetically significant alterations in breast cancer cells in Bin1.

Conclusions

The studies described above that were performed in year one support the conclusion that Bin1 is normally expressed in breast cells but is frequently lost in tumor cells. This loss may reflect gene deletion, as we have seen an example of this, but more frequently may reflect either missense or nonsense mutations as well as epigenetic mechanisms that shut off gene expression (e.g. methylation). A further conclusion of this year's work is that Bin1 can inhibit malignant cell growth via both Myc-dependent and Myc-independent mechanisms, thus extending its anti-proliferative potential in breast cancer cells. The analysis of Bin1 performed during this line of work sets the stage for a functional analysis of breast cancer cell mutations anticipated to be identified this year in our panel of 22 tumors, as the work progresses.

References

- Bello-Fernandez, C., Packham, G. and Cleveland, J.L. (1993). The ornithine decarboxylase gene is a transcriptional target of c-Myc. *Proc Natl Acad Sci USA* **90**, 7804-8.
- Berns, E.M., Klijn, J.G., van, P.W., van, S.I., Portengen, H. and Foekens, J.A. (1992). c-myc amplification is a better prognostic factor than HER2/neu amplification in primary breast cancer. *Cancer Res* **52**, 1107-13.
- Borg, A., Baldetorp, B., Ferno, M., Olsson, H. and Sigurdsson, H. (1992). c-myc amplification is an independent prognostic factor in postmenopausal breast cancer. *Int J Cancer* **51**, 687-91.
- Hehir, D.J., McGreal, G., Kirwan, W.O., Kealy, W. and Brady, M.P. (1993). c-myc oncogene expression: a marker for females at risk of breast carcinoma. *J Surg Oncol* **54**, 207-9.
- Kang, Y., Cortina, R. and Perry, R.R. (1996). Role of c-myc in tamoxifen-induced apoptosis in estrogen-independent breast cancer cells. *J Natl Cancer Inst* **88**, 279-84.
- Manni, A., Wechter, R., Wei, L., Heitjan, D. and Demers, L. (1995). Phenotypic features of breast cancer cells overexpressing ornithine-decarboxylase. *J Cell Physiol* **163**, 129-36.
- Prendergast, G.C. and Cole, M.D. (1989). Posttranscriptional regulation of cellular gene expression by the c-myc oncogene. *Mol Cell Biol* **9**, 124-34.
- Prendergast, G.C., Diamond, L.E., Dahl, D. and Cole, M.D. (1990). The c-myc-regulated gene *mrl* encodes plasminogen activator inhibitor 1. *Mol Cell Biol* **10**, 1265-9.
- Reilly, D., Christensen, L., Duch, M., Nolan, N., Duffy, M.J. and Andreasen, P.A. (1992). Type-1 plasminogen activator inhibitor in human breast carcinomas. *Int J Cancer* **50**, 208-14.
- Sakamuro, D., Elliott, K., Wechsler-Reya, R. and Prendergast, G.C. (1996). BIN1 is a novel MYC-interacting protein with features of a tumor suppressor. *Nature Genet* **14**, 69-77.
- Shiu, R.P., Watson, P.H. and Dubik, D. (1993). c-myc oncogene expression in estrogen-dependent and -independent breast cancer. *Clin Chem* **39**, 353-5.
- Sumiyoshi, K., Serizawa, K., Urano, T., Takada, Y., Takada, A. and Baba, S. (1992). Plasminogen activator system in human breast cancer. *Int J Cancer* **50**, 345-8.
- Watson, P.H., Safneck, J.R., Le, K., Dubik, D. and Shiu, R.P. (1993). Relationship of c-myc amplification to progression of breast cancer from in situ to invasive tumor and lymph node metastasis. *J Natl Cancer Inst* **85**, 902-7.
- Wechsler-Reya, R., Sakamuro, D., Zhang, J., DuHadaway, J. and Prendergast, G.C. (1997). Structural analysis of the human BIN1 gene: evidence for alternate RNA splicing and tissue-specific regulation. *J Biol Chem*, in press.

Appendices (publication reprints and preprints)

- Sakamuro, D, Elliott, K., Wechsler-Reya, R., and Prendergast, G.C. (1996). BIN1 is a novel MYC-interacting protein with features of a tumor suppressor. *Nature Genet* **14**, 69-77.
- Wechsler-Reya, R., Elliott, K., Herlyn, M., and Prendergast, G.C. (1997). The putative tumor suppressor BIN1 is a short-lived nuclear phosphoprotein whose localization is altered in malignant cells. *Cancer Res* **57**, 3258-3263.
- Wechsler-Reya, R., Sakamuro, D., Zhang, J., and Prendergast, G.C. (1997). Structural analysis of the human BIN1 gene: evidence for alternate splicing and tissue-specific regulation. *J Biol Chem*, in press.
- Elliott, K., Sakamuro, D., Du, W., and Prendergast, G.C. BIN1 inhibits MYC transactivation and suppresses malignant cell proliferation via MYC-dependent and MYC-independent mechanisms. *Oncogene*, under revision.

The Putative Tumor Suppressor BIN1 Is a Short-Lived Nuclear Phosphoprotein, the Localization of Which Is Altered in Malignant Cells¹

Robert Wechsler-Reya,² Katherine Elliott, Meenhard Herlyn, and George C. Prendergast³

The Wistar Institute, Philadelphia, Pennsylvania 19104

ABSTRACT

BIN1 is a putative tumor suppressor that was identified in a genetic screen for polypeptides that interact with the MYC oncoprotein. Using a set of six monoclonal antibodies, we identified and examined biochemical features and localization of cellular BIN1. Epitope mapping indicated that a putative nuclear localization motif and the MYC-binding domain were among the regions recognized by five antibodies. In immunoprecipitation and Western analyses, cellular BIN1 was identified in human and rodent cells as a monomeric phosphoprotein of $M_r \sim 70,000$. Pulse-chase experiments showed that BIN1 was short-lived, with a half-life of ~ 2 h. Cell immunofluorescence experiments revealed overlapping but unique nuclear localization patterns distinguished by two different antibodies. In normal cells, BIN1 was predominantly nucleoplasmic but was also present in a subnuclear compartment. Conversely, in a panel of tumor cells that expressed BIN1, the predominant localization was the subnuclear compartment. Taken together, the results suggested that the antibodies recognized different isoforms or conformations of BIN1, the localization of which varied between normal and tumor cells. This study will facilitate further analysis of the structure and regulation of BIN1 in normal and malignant cells.

INTRODUCTION

The identification and analysis of tumor suppressors is of major importance for improved diagnosis and treatment of solid tumors. BIN1 is a novel protein that has features of a tumor suppressor in carcinoma of the breast, prostate, and liver (1). It was originally identified through its interaction with the MYC oncoprotein, which has a major role in many human cancers (2-4). A role for BIN1 in controlling neoplastic cell growth and cell cycle transit has been suggested by several observations. First, BIN1 inhibits malignant cell transformation by both MYC and the adenovirus E1A protein (1). Second, BIN1 is related to amphiphysin, a neuronal protein that is the autoimmune target of paraneoplastic disorders associated with breast and lung cancer (5, 6), and to RVS167, a negative regulator of the cell cycle in yeast (7). Third, although widely expressed in normal cells, BIN1 is poorly expressed or undetectable in $\sim 50\%$ of carcinoma cell lines and primary breast carcinomas examined (1). Fourth, deficits in expression are functionally significant, because ectopic BIN1 can inhibit the growth of tumor cells that lack endogenous expression (1). Finally, the human *BIN1* gene has been mapped to chromosome 2q14 (8), within a mid-2q region that is deleted in $\sim 42\%$ of metastatic prostate cancers (9), and at the syntenic murine locus, in $>90\%$ of radiation-induced myeloid leukemias (10).

Taken together, these results have prompted the hypothesis that BIN1 is a tumor suppressor, the loss of which may contribute to

growth deregulation in cancer cells. A prerequisite for testing this hypothesis is biochemical analysis of cellular BIN1, which has been poorly characterized. A polyclonal antiserum that was generated previously recognized a BIN1-related polypeptide as well as BIN1 (1), so a more specific reagent was sought. Here, we report the characterization of a set of mAbs⁴ that identify cellular BIN1 as a short-lived nuclear phosphoprotein the localization of which is altered in malignant cells.

MATERIALS AND METHODS

Hybridoma Production. BALB/c mice were immunized with the BIN1 recombinant fusion protein GST-99Pst (1). Hybridomas were generated using the nonsecreting murine myeloma T3X63Ag8.Sp2/0 and by ELISA as described (11). All anti-BIN1 mAbs were of the IgG1 κ isotype, and the hybridomas secreted 5-10 μ g/ml IgG into the culture supernatant.

Plasmids. BIN1 deletion mutants were generated by standard PCR methodology, except where indicated. Inserts were subcloned for expression into the vector pcDNA3 (Invitrogen). Full-length BIN1 and BIN1 Δ MBD have been described (1). BIN1 Δ BAR-C was generated by dropping an internal *A*/III restriction fragment from full-length BIN1, resulting in a deletion of amino acids 125-207 from the BAR domain (1). The remaining mutants were generated by standard PCR methodology using the oligonucleotide primers 995'(Bam), 993'SH3(Xho) (1), and others, the sequence of which is derived from the BIN1 cDNA sequence (GenBank accession no. U68485). BIN1 Δ U1 lacks amino acids 224-248; BIN1 Δ NLS, amino acids 251-269; BIN1 Δ SH3, amino acids 384-451; the other mutants lack the residues indicated. The integrity of PCR-generated fragments was verified by DNA sequencing.⁵

IP. IPs were performed in NP40 buffer essentially as described (12). ³⁵S-methionine-labeled BIN1 mutant polypeptides were generated by IVT according to the protocol provided by the vendor (Promega). For IP, 10 μ l of an IVT reaction were mixed with 100 μ l of hybridoma supernatant and 20 μ l of protein G-Sepharose beads (Pharmacia) in 1 ml NP40 lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1% NP-40] and incubated 1 h at 4°C on a nutator shaker. Polypeptides bound to beads were washed four times with the same buffer, resuspended in 2 \times SDS-PAGE gel loading buffer, boiled for 3 min, fractionated on 10% SDS polyacrylamide gels, and fluorographed.

C2C12 myoblasts (a gift of D. Goldhamer, University of Pennsylvania, Philadelphia, PA) were cultured in DMEM containing 15% fetal bovine serum and penicillin/streptomycin. Cells were labeled for 4 h in growth medium lacking methionine and cysteine (Life Technologies, Inc.) with 100 μ Ci/ml EXPRESS label (NEN) or in growth medium lacking phosphate (Life Technologies, Inc.) with 1 mCi/ml ³²P-orthophosphate. Lysates prepared in NP40 buffer and phenylmethylsulfonyl fluoride, leupeptin, and aprotinin were centrifuged for 15 min at maximum speed in a microcentrifuge chilled to 4°C. Extract protein was precleared by a 1-h incubation at 4°C with 20 μ l of protein G-Sepharose beads. Complexes formed after IP of 0.5 mg of precleared extract with 100 μ l of hybridoma supernatant in 1 ml of NP40 buffer were collected on 20 μ l of protein G-Sepharose beads and analyzed as above. Nonreducing conditions were maintained in one experiment by omitting DTT from all gel loading and preparation buffers. For the phosphatase experiment, immunoprecipitates were treated for 15 min with 10 units of calf intestinal alkaline phosphatase (Boehringer-Mannheim) before SDS-PAGE and fluorography.

Received 2/10/97; accepted 5/23/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported by American Cancer Society Grant CN-160 and United States Army Breast Cancer Research program Grant DAMD17-96-1-6324 (to G. C. P.). R. W.-R. is a Medical Research Council of Canada Postdoctoral Fellow. K. E. was supported by a NIH Training Grant. G. C. P. is the recipient of an ACS Junior Faculty Award and is a Pew Scholar in the Biomedical Sciences.

² Present address: Department of Neurobiology, Fairchild Building, Room D249, Stanford University School of Medicine, Stanford, CA 94305.

³ To whom requests for reprints should be addressed, at The Wistar Institute, 3601 Spruce Street, Philadelphia, PA 19104.

⁴ The abbreviations used are: mAb, monoclonal antibody; MBD, MYC-binding domain; IP, immunoprecipitation; IVT, *in vitro* translation; NLS, nuclear localization signal; BAR, BIN1/amphiphysin/RVS167-related.

⁵ Oligonucleotides and details for each construction were omitted to conserve space and are available from G. C. P.

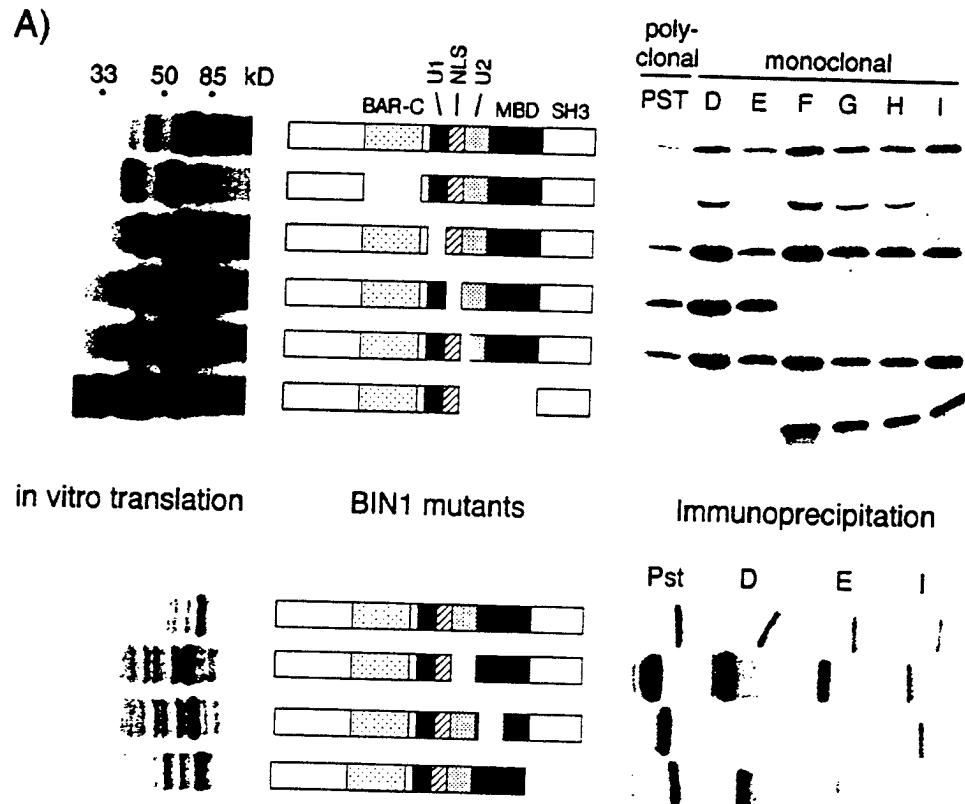
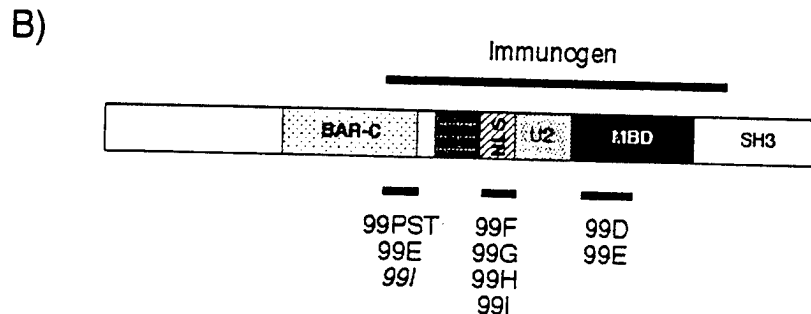


Fig. 1. Epitope mapping of BIN1 antibodies. A. IP. A set of ³⁵S-labeled BIN1 deletion mutants expressed by IVT were analyzed by SDS-PAGE and fluorography (left) or immunoprecipitated with the indicated BIN1 antibodies before similar analyses (right). The schematics (center) indicate the regions deleted from full-length BIN1 (1), including the BAR-C, U1, NLS, U2, MBD, and SH3 domains. The deletions tested were BIN1ΔBAR-C, BIN1ΔU1, BIN1ΔNLS, BIN1Δ270-288, BIN1ΔMBD (top section) and BIN1Δ270-315, BIN1Δ323-356, and BIN1ΔSH3 (bottom section). B. epitope locations. The region of BIN1 included in the immunogen and the regions required for IP by each antibody are shown.



Western Blotting. Cell extracts were prepared in NP40 buffer from human HepG2, MCF7, WI-38, and HeLa cells obtained from the American Type Culture Collection cultured as described above. Fifty μ g of cell protein were fractionated on 10% SDS-polyacrylamide gels, transferred to Hybond ECL nitrocellulose (Amersham Corp.), and processed using a 1:20 dilution of hybridoma supernatant using standard methods (12). Blots were probed with a 1:1000 dilution of horseradish peroxidase-conjugated antimouse IgG (Boehringer-Mannheim) for the secondary antibody and developed using a chemiluminescence kit (Pierce), following a protocol provided by the vendor.

Pulse-Chase Analysis. COS cells cultured as above were transfected as described (13) with 20 μ g of the BIN1 expression plasmid CMV-99E (1). Thirty-six h later, cells were pulse-labeled for 30 min with 100 μ Ci/ml EXPRESS label (NEN) in growth medium lacking methionine and cysteine (Life Technologies, Inc.), washed twice with PBS, and re-fed with normal growth medium. After 0, 2, 5, or 10 h, cell extracts were prepared and processed for IP, SDS-PAGE, and fluorography as described above.

Cellular Immunofluorescence. Rat1 fibroblasts, human SAOS-2 osteosarcoma cells, LNCaP prostate carcinoma cells, or HeLa cells were seeded into the same growth medium as above on glass coverslips. After overnight incubation, cells were processed for indirect immunofluorescence using a 1:20 dilution of hybridoma supernatants essentially as described (14). Cells were fixed with 1% paraformaldehyde/PBS, permeabilized with 0.2% Triton X-100/

PBS, incubated with primary BIN1 mAb in 0.1% Triton X-100/PBS for 1 h followed by a secondary fluorescein-conjugated antimouse IgG antibody (Boehringer-Mannheim), mounted in Fluoromount G (Southern Biotechnology), and viewed on a Leica immunofluorescence microscope. Photographic exposures were 2-5 s for 99D and 10-15 s for 99E.

RESULTS

Epitope Mapping of a Set of BIN1 Monoclonal Antibodies. To generate mAbs specific for BIN1, mice were immunized with GST-99Pst, a glutathione S-transferase fusion polypeptide containing amino acids 189-398 of human BIN1 (1). Screening for GST-99Pst-reactive immunoglobulins in hybridoma supernatants was performed by ELISA. Six hybridomas that were strongly positive for the GST-99Pst immunogen and negative for unfused GST (designated 99D through 99I) were characterized further.

To map the epitopes recognized by each of the antibodies, hybridoma supernatants were assayed for the ability to immunoprecipitate BIN1 and a variety of BIN1 deletion mutants (a detailed description of the mutants will appear elsewhere⁷). IPs were performed as de-

⁶ K. Elliott and G. C. Prendergast, unpublished results.

⁷ K. Elliott, R. Wechsler-Reya, and G. C. Prendergast, manuscript in preparation.

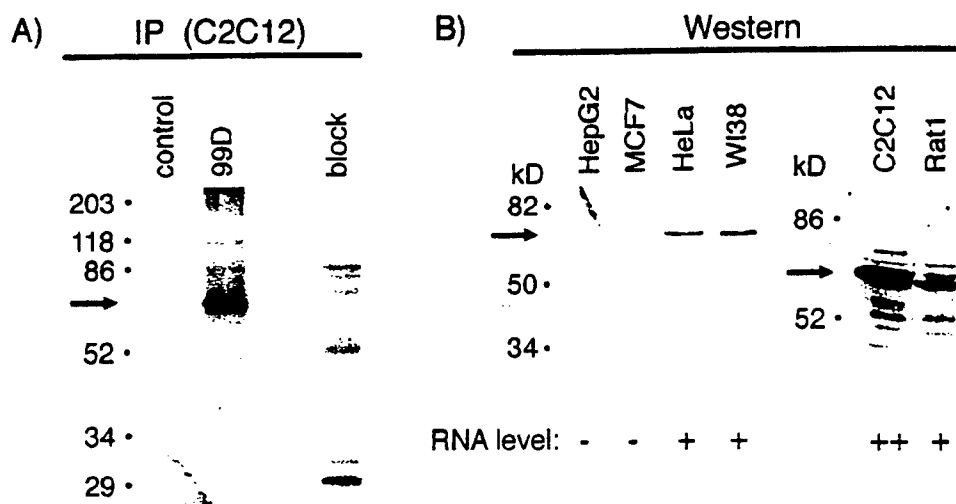


Fig. 2. Recognition of a $M_r \sim 70,000$ cellular BIN1 polypeptide in human and rodent cells. A. detection of BIN1 in C2C12 cells by IP. Proteins immunoprecipitated by 99D from ^{35}S -labeled C2C12 cell extracts were subjected to SDS-PAGE and fluorography. Control, murine anti-IgD was used added instead of 99D; block, 99D was preincubated with immunogen before IP. B. Western analysis of BIN1 in human and rodent cell lines. A Western blot of extracts from the cell lines indicated was probed with mAb 99D (left). The relative level of expression of BIN1 RNA in each line is indicated below the blots.

scribed in the "Materials and Methods." Briefly, supernatants were mixed with ^{35}S -methionine-labeled polypeptides generated by IVT, and immune complexes were precipitated with protein G-agarose. Precipitates were then washed four times in IP buffer, fractionated by SDS-PAGE, and fluorographed. A polyclonal rabbit serum (anti-99Pst) raised against the same immunogen was included as a positive control. In control experiments, none of the antibodies reacted with any of several nonspecific IVT products tested (data not shown). However, as shown in Fig. 1A, each of the mAbs precipitated BIN1 and the majority of the deletion mutants tested.

The mutants not precipitated by a given antibody provided information about the region(s) of BIN1 required for binding by that antibody. The region required for binding of 99D mapped to a 33-residue segment of the MBD (amino acids 323–356). The region bound by 99F, 99G, and 99H mapped to the putative NLS located at amino acids 251–269. Recognition by 99I also required the NLS; however, this antibody inefficiently precipitated a mutant lacking BAR-C, a region that comprises approximately the COOH-terminal half of the BAR domain of BIN1 (1). This result suggested that BAR-C may contribute to or affect recognition of the 99I epitope. 99E appeared to recognize a noncontiguous epitope that included elements in BAR-C and the 33-amino acid MBD segment. The regions required for recognition by 99E and the anti-99Pst antiserum were similar, except that anti-99Pst required a more COOH-terminal region of the MBD than did 99E. The epitopes in BAR-C could be mapped to a 17-amino acid segment between amino acids 189 and 206 because this was the only part of BAR-C included in the GST-99Pst immunogen (1). A summary of the regions required for recognition by each antibody is shown in Fig. 1B. We concluded that at least three separate epitopes located within the MBD, NLS, and BAR-C regions of BIN1 were recognized by this set of antibodies.

Cellular BIN1 Is a Monomeric Phosphoprotein of $M_r \sim 70,000$.

In a series of Western blotting experiments with bacterially expressed polypeptides, 99D exhibited the highest sensitivity and specificity of the antibodies generated (data not shown). Therefore, 99D was the chief reagent used to identify and characterize BIN1 by Western analysis and IP. We examined two human lines that express the BIN1 message, WI-38 diploid fibroblasts and HeLa cervical carcinoma cells, and two that lack it, HepG2 hepatocarcinoma and MCF7 breast carcinoma cells (1). We also examined two rodent cell lines, C2C12 and Rat1. C2C12 is a skeletal myoblast line (15) that was included as a positive control because BIN1 had been observed to be highly expressed in murine skeletal muscle (1). Rat1 is an immortalized and serum-regulated fibroblast line useful for cell growth and transformation experiments.

To test the ability of 99D to recognize cellular BIN1, C2C12 cells were labeled with ^{35}S -methionine/cysteine and lysed in NP40 buffer. Lysates were then subjected to IP with 99D or an isotype-matched control antibody (anti-IgD). As shown in Fig. 2A, 99D (but not anti-IgD) precipitated a protein with a molecular weight of $\sim 70,000$, consistent with the size of the polypeptide encoded by a full-length cDNA (1). The band detected was specific because preincubation of 99D with unlabeled GST-99Pst prevented its appearance on the gel. Moreover, a band of similar size was detected by Western blot analysis in WI-38, HeLa, and Rat1 cells, which express BIN1 mRNA, but not in MCF7 or HepG2 cells, which do not (Fig. 2B). 99D did not recognize by either IP or Western blotting a $M_r \sim 45,000$ Bin1-related polypeptide that was detected previously with a polyclonal antiserum (1). Taken together, the results suggested that BIN1 was a $M_r \sim 70,000$ polypeptide that could be detected in both human and rodent cells.

We examined the biochemical characteristics of cellular BIN1 in C2C12 cells, which express high levels of the protein. Because the sequence of BIN1 includes three cysteines, we determined first whether intermolecular disulfide bonds might stabilize oligomeric forms of BIN1 *in vivo*. Cell lysates and BIN1 immunoprecipitates were fractionated on either nonreducing or reducing gels. Western blotted, and probed with 99D. As seen in Fig. 3A, the mobility of BIN1 was similar in both gels, indicating that BIN1 did not undergo disulfide-mediated oligomerization *in vivo*. We concluded that cellular BIN1 was a $M_r \sim 70,000$ monomeric polypeptide.

Analysis of the BIN1 amino acid sequence with the algorithm PPSEARCH/PROSITE revealed several consensus sites for serine/threonine or tyrosine phosphorylation (data not shown). Therefore, to determine whether BIN1 is a phosphoprotein, we immunoprecipitated BIN1 from extracts of C2C12 cells that had been metabolically labeled with either ^{32}P -orthophosphate or ^{35}S -methionine/cysteine. A specific $M_r \sim 70,000$ band was detected in extracts derived from each labeling reaction (see Fig. 3B, top), arguing that BIN1 was indeed phosphorylated *in vivo*. Interestingly, in ^{32}P -labeled extracts, at least four less abundant polypeptides of $M_r \sim 200,000$, 110,000, 90,000, 80,000, and 60,000 were also observed. These polypeptides were specifically immunoprecipitated because their appearance was blocked by preincubation of the antibody with immunogen. Their absence from Western blots suggested that they might represent proteins that coprecipitate with BIN1. To confirm that the $M_r \sim 70,000$ band represented a phosphorylated species, immunoprecipitates from ^{35}S - and ^{32}P -labeled C2C12 cells were incubated with calf intestinal phosphatase before SDS-PAGE. This treatment reduced the intensity of the ^{32}P -labeled but not the ^{35}S -labeled BIN1 species (see Fig. 3B).

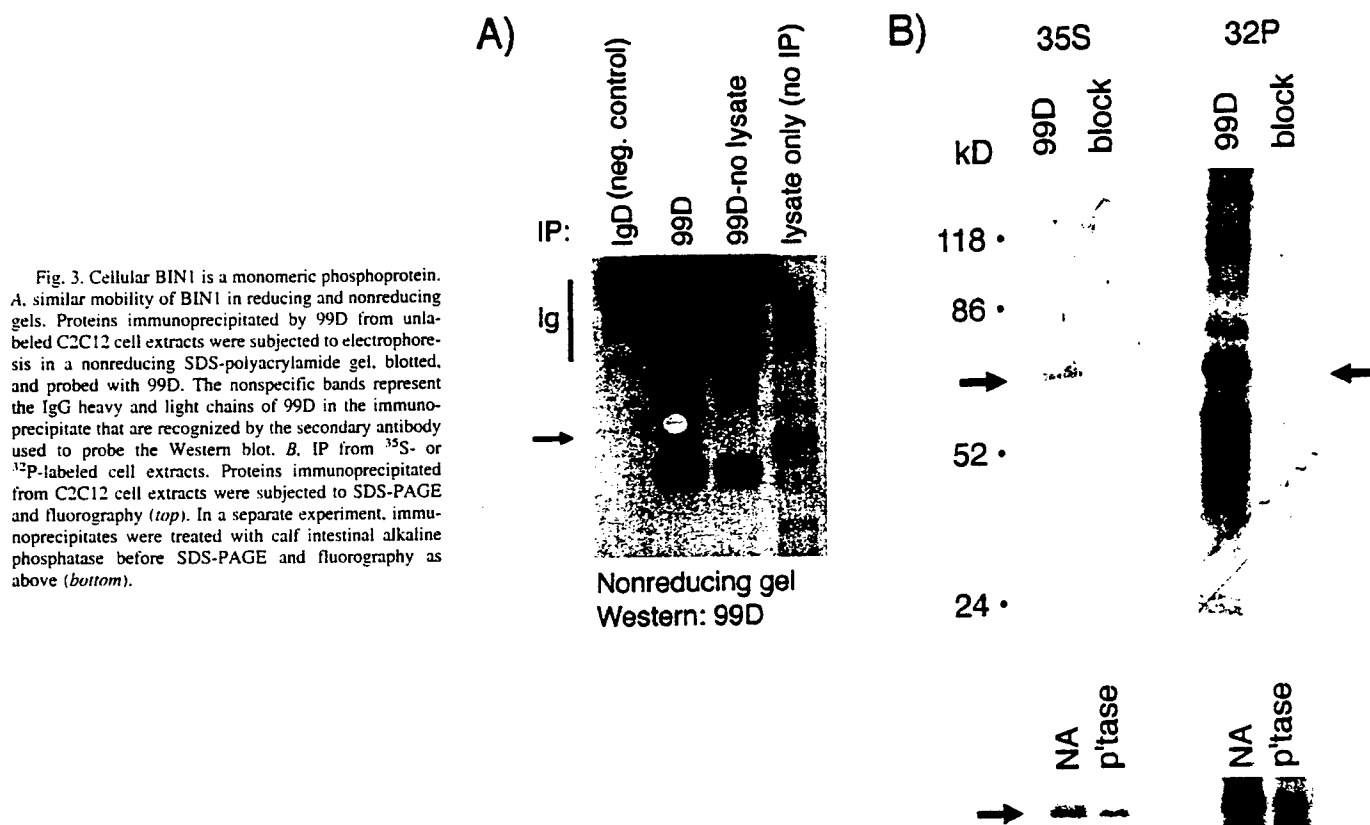


Fig. 3. Cellular BIN1 is a monomeric phosphoprotein. A, similar mobility of BIN1 in reducing and nonreducing gels. Proteins immunoprecipitated by 99D from unlabeled C2C12 cell extracts were subjected to electrophoresis in a nonreducing SDS-polyacrylamide gel, blotted, and probed with 99D. The nonspecific bands represent the IgG heavy and light chains of 99D in the immunoprecipitate that are recognized by the secondary antibody used to probe the Western blot. B, IP from ³⁵S- or ³²P-labeled cell extracts. Proteins immunoprecipitated from C2C12 cell extracts were subjected to SDS-PAGE and fluorography (top). In a separate experiment, immunoprecipitates were treated with calf intestinal alkaline phosphatase before SDS-PAGE and fluorography as above (bottom).

bottom). We concluded that cellular BIN1 was a monomeric phosphoprotein.

Cellular BIN1 Is Short-Lived. BIN1 was initially identified through its interaction with the putative transactivation domain of the MYC oncoprotein (1). MYC is induced as quiescent cells enter the cell cycle and has a half-life of only 15–20 min *in vivo* (16–19). Therefore, it was of interest to determine whether BIN1 was growth regulated and/or short lived. To address the first issue, Northern and Western analyses were performed using RNA and protein isolated from Rat1 fibroblasts that were proliferating, growth arrested at confluence, or quiescent and induced to enter the cell cycle by serum stimulation. The results indicated that BIN1 mRNA and protein were constitutively expressed and that their steady-state levels did not vary during the cell cycle (data not shown).

To examine the half-life of BIN1, a pulse-chase experiment was performed in transiently transfected COS cells. Briefly, cells transfected with a BIN1 expression vector were pulse labeled for 30 min with ³⁵S-methionine/cysteine and then lysed immediately (pulse) or washed and incubated for 2, 5, or 10 h in normal growth medium before lysis (chase). Lysates from each time point were subjected to IP with 99D and BIN1 labeling was assessed by SDS-PAGE and fluorography. We observed a ~2-fold reduction in the intensity of the BIN1 band at each time point, indicating that BIN1 had a half-life of ~2 h (see Fig. 4).

Distinct Nuclear Localizations of BIN1 in Normal and Tumor Cells. To examine the localization of BIN1 in cells, we conducted immunofluorescence experiments in normal Rat1 cells using 99D, 99E, and 99F, which represented the three epitope specificities present among the six mAbs generated. We did not observe cell staining with 99F, suggesting that the NLS epitope was masked or absent from BIN1 species in Rat1 cells under the experimental conditions used for detection. In contrast, 99D and 99E recognized overlapping but distinct localizations of BIN1 in the nucleus (see Fig. 5A). Cells stained

with 99D displayed fluorescence in the nucleoplasm. A similar pattern was observed in C2C12 murine myoblasts and IMR-90 human diploid fibroblasts (two "normal" cell types) and in cells in sections of normal colon and breast tissue (data not shown). In contrast, cells stained with 99E showed fluorescence in a punctate subnuclear domain. The patterns observed with each of these antibodies were specific, because their appearance was blocked by preincubating the antibodies with immunogen. The punctate pattern produced by 99E was similar to that observed previously using anti-99Pst to stain BIN1-transfected HepG2 cells (which lack endogenous BIN1 or BIN1-related proteins; Ref. 1), consistent with the fact that the epitopes recognized by each antibody

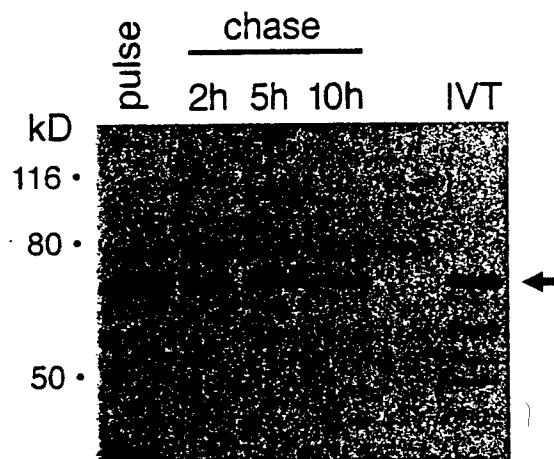


Fig. 4. Cellular BIN1 has a ~2-h half-life. COS cells were transiently transfected with a BIN1 expression vector. Thirty-six h later, cells were labeled for 30 min with ³⁵S-methionine/cysteine, and the label was chased by the addition of normal growth medium. After the times indicated, cell extracts were prepared and processed for IP, SDS-PAGE, and fluorography.

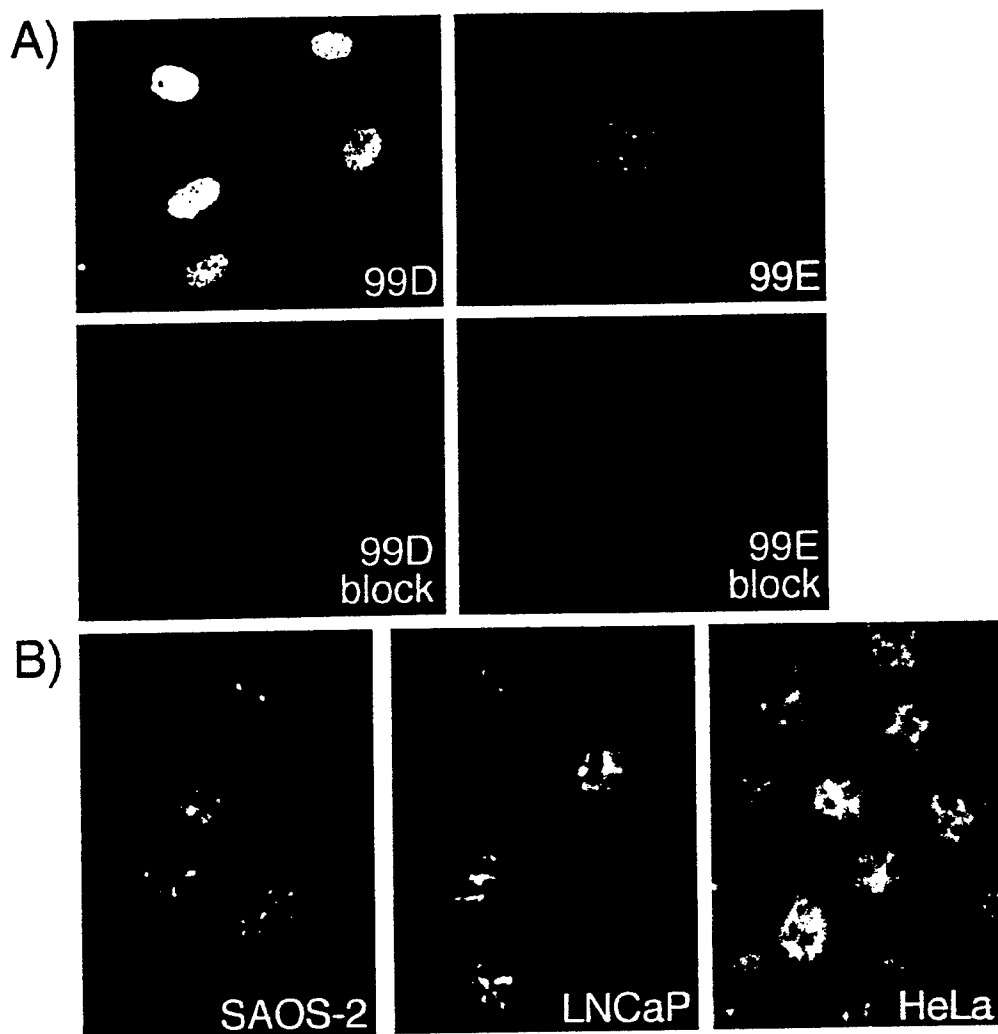


Fig. 5. BIN1 is localized in the nucleoplasm or a subnuclear domain of normal proliferating or tumor cells. A, detection of BIN1 in normal cells. Rat1 fibroblasts were processed for indirect immunofluorescence with 99D or 99E. The magnification in the 99E panel is higher to illustrate the fine punctate pattern seen. *Block*, antibodies were preincubated with immunogen before use. B, detection of BIN1 in tumor cells that express endogenous BIN1. SAOS-2, LNCaP, and HeLa cells were processed for indirect immunofluorescence with 99D.

were related. Therefore, although the basis for the staining difference between 99D and 99E was not entirely clear, the similarity between 99E and anti-99Pst supported the conclusion that the punctate pattern was not artifactual. These results indicated that 99D and 99E identified BIN1 species that could be distinguished in cells by differences in their nuclear localization.

Previous results indicated that BIN1 expression is lacking or altered at the level of RNA in ~50% of carcinoma cells examined (1). In tumor cells that expressed BIN1 RNA, we wished to examine the localization of BIN1 protein because of the possibility that altered localization patterns may have consequences for protein function. Therefore, we stained HeLa cervical carcinoma, SAOS-2 osteosarcoma, and LNCaP prostate carcinoma cells, each of which had been demonstrated to express the BIN1 message (1).³ In each cell line, both nucleoplasmic and subnuclear punctate staining was observed (see Fig. 5B). In SAOS-2 and LNCaP cells, the punctate pattern predominated; in HeLa cells, the two compartments were stained more equally. However, in each case, the localization pattern varied significantly from that observed in proliferating Rat1 cells, where nucleoplasmic staining clearly predominated. Taken together, the results indicated that different isoforms or conformations of BIN1 were associated with different subnuclear localizations in normal or tumor cells.

DISCUSSION

In this study, we characterized a set of BIN1 monoclonal antibodies and used these reagents to identify cellular BIN1 as a short-lived nuclear phosphoprotein. In addition, we found that antibodies recognizing different epitopes stained BIN1 species that were located in different nuclear compartments and that were expressed preferentially in normal or tumor cells.

In a series of Western blotting and IP experiments, we identified cellular BIN1 as a monomeric phosphoprotein with an apparent molecular weight of ~70,000. Although larger than its predicted molecular weight (50,000), the size of cellular BIN1 was consistent with the size of a full-length *BIN1* cDNA expressed in COS cells, which migrates aberrantly in SDS gels due to a MBD determinant (1). In addition, the abundance of cellular BIN1 correlated well with the level of steady-state message in each of the cell lines examined. These observations, together with the fact that its precipitation could be blocked by preincubation of the precipitating mAb with immunogen, suggested that the protein was in fact cellular BIN1. In pulse-chase experiments, we found that the half-life of BIN1 was ~2 h, approaching that of MYC, which is approximately 30 min (16–19). Although a relatively rapid turnover rate would allow BIN1 levels to be tightly regulated, we found that the steady-state level of BIN1 did not vary in normal Rat1 cells that were proliferating, quiescent, or induced to

³ K. Elliott, D. Sakamuro and G. C. Prendergast, unpublished results.

enter the cell cycle by growth factor stimulation. The fact that BIN1 is phosphorylated *in vivo* raises the possibility that its function(s) may be regulated by changes in phosphorylation. If so, the formation or function of a MYC-BIN1 complex might be regulated posttranslationally. In future studies, it will be necessary to examine this issue, as well as to determine whether the phosphorylation status of BIN1 affects its MYC-dependent or MYC-independent growth-inhibitory activity (1).

The antibodies developed for this study recognized at least three distinct epitopes in BIN1. 99D recognized a MBD epitope and was a "pan" antibody in the sense that it recognized BIN1 species in the nucleoplasm and a subnuclear punctate compartment in several cell types. Because it recognizes a region of the MBD, 99D may be useful to inhibit BIN1 interaction with MYC *in vitro* or *in vivo*. 99E recognized a noncontiguous epitope composed of BAR-C and MBD elements that was detected only on cellular BIN1 species located in a subnuclear compartment. The contribution of BAR-C and MBD elements to the epitope suggested that these regions may be proximal to each other in certain forms of native BIN1. 99F recognized a NLS epitope that was not detected in cellular BIN1 species in Rat1 cells, suggesting that the NLS region of the protein might be obscured or missing in these cells. Interestingly, we have found recently that 99F recognizes a cytoplasmic form of BIN1 in C2C12 myoblasts induced to differentiate by serum withdrawal.⁹ The nature of this polypeptide and its relationship to the forms of BIN1 observed in proliferating C2C12 cells that are not recognized by 99F are currently under investigation.

The differences in staining observed with each of these antibodies suggest that several forms of BIN1 may be present in cells. One possible explanation for this is that BIN1 adopts different conformations (which mask or reveal certain epitopes) at different cell loci. However, we have recently discovered that BIN1 is subjected to alternate splicing.¹⁰ This raises the possibility that the different staining patterns represent different BIN1 isoforms that lack or include the epitopes required for binding by particular antibodies.

An unexpected finding of this study was that the localization of cellular BIN1 varies significantly in normal and malignant cells. In normal cells, where growth is regulated, BIN1 is located primarily in the nucleoplasm, but a fraction of the protein is located in a subnuclear punctate compartment(s). In tumor cells, where growth is deregulated, the subnuclear punctate localization predominates. The basis for the subnuclear staining pattern is unclear. The dot-like structures do not represent nucleoli, which can be readily distinguished by their lack of staining (*e.g.*, see SAOS-2 cells in Fig. 5B). Several other subnuclear domains have been characterized (reviewed in Ref. 20). Two such domains that may be germane to BIN1 and its growth-inhibitory activities are the ND10 domain (21), which is reorganized by viral proteins that induce DNA replication (22–24), and a BRCA1/RAD51-associated domain, which is subject to cell cycle regulation (25–28). Whatever the basis for the different staining patterns observed in normal and tumor cells, these observations raise the possibility that there is a correlation between the localization and growth regulatory capacity of BIN1. In future work, it will be important to define the basis for the various localization patterns identified and determine whether they have different consequences for MYC-dependent and MYC-independent growth-inhibition activities (1).

ACKNOWLEDGMENTS

We thank R. Buccafusca for technical assistance, D. Goldhamer for C2C12 myoblasts, and S. Sukoff (Wistar Hybridoma Core Facility). For discussion and criticism we thank G. Maul, U. Rodeck, and members of our laboratory.

REFERENCES

1. Sakamuro, D., Elliott, K., Wechsler-Reya, R., and Prendergast, G. C. BIN1 is a novel MYC-interacting protein with features of a tumor suppressor. *Nat. Genet.* 14: 69–77, 1996.
2. Cole, M. D. The *myc* oncogene: its role in transformation and differentiation. *Ann. Rev. Genet.* 20: 361–384, 1986.
3. Cory, S. Activation of cellular oncogenes in hematopoietic cells by chromosome translocation. *Adv. Cancer Res.* 47: 189–234, 1986.
4. De Pinho, R. A., Schreiber-Agus, N., and Alt, F. W. *myc* family oncogenes in the development of normal and neoplastic cells. *Adv. Cancer Res.* 57: 1–46, 1991.
5. David, C., Solimena, M., and De Camilli, P. Autoimmunity in Stiff-Man Syndrome with breast cancer is targeted to the C-terminal regulation of human amphiphysin, a protein similar to the yeast proteins, Rvs161 and Rvs167. *FEBS Lett.* 351: 73–79, 1994.
6. Dropcho, E. J. Anti-amphiphysin antibodies with small-cell lung carcinoma and paraneoplastic encephalomyelitis. *Ann. Neurol.* 39: 659–667, 1996.
7. Bauer, F., Urdaci, M., Aigle, M., and Crouzet, M. Alteration of a yeast SH3 protein leads to conditional viability with defects in cytoskeletal and budding patterns. *Mol. Cell. Biol.* 13: 5070–5084, 1993.
8. Negorev, D., Reithman, H., Wechsler-Reya, R., Sakamuro, D., Prendergast, G. C., and Simon, D. The *Bin1* gene localizes to human chromosome 2q1.4 by PCR analysis of somatic cell hybrids and fluorescence *in situ* hybridization. *Genomics* 33: 329–331, 1996.
9. Cher, M. L., Bova, G. S., Moore, D. H., Small, E. J., Carroll, P. R., Pin, S. S., Epstein, J. I., Isaacs, W. B., and Jensen, R. H. Genetic alterations in untreated metastases and androgen-independent prostate cancer detected by comparative genomic hybridization and allelotyping. *Cancer Res.* 56: 3091–3102, 1996.
10. Hayata, I., Seki, M., Yoshida, K., Hirashima, K., Sado, T., Yamagiwa, J., and Ishihara, T. Chromosomal aberrations observed in K2 mouse myeloid leukemias. *Cancer Res.* 43: 367–373, 1983.
11. Koprowski, H., Steplewski, Z., Mitchell, K., Herlyn, M., Herlyn, D., and Fuhrer, P. Colorectal carcinoma antigens detected by hybridoma antibodies. *Somatic Cell Genet.* 5: 957–972, 1979.
12. Harlow, E., and Lane, D. *Antibodies: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1988.
13. Margolin, J. F., Friedman, J. R., Meyer, W. K., Vissing, H., Thiesen, H. J., and Rauscher, F. J. Kruppel-associated boxes are potent transcriptional repressor domains. *Proc. Natl. Acad. Sci. USA* 91: 4509–4513, 1994.
14. Prendergast, G. C., Hopewell, R., Gorham, B., and Ziff, E. B. Biphasic effect of Max on Myc transformation activity and dependence on N- and C-terminal Max functions. *Genes Dev.* 6: 2429–2439, 1992.
15. Blau, H. M., Pavlath, G. K., Hardeman, E. C., Chiu, C. P., Silberstein, L., Webster, S. G., Miller, S. C., and Webster, C. Plasticity of the differentiated state. *Science (Washington DC)* 230: 758–766, 1985.
16. Beimling, P., Benter, T., Sander, T., and Moelling, K. Isolation and characterization of the human cellular *myc* gene product. *Biochemistry* 24: 6349–6355, 1985.
17. Hann, S. R., Thompson, C. B., and Eisenman, R. N. *c-myc* protein synthesis is independent of the cell cycle in human and avian cells. *Nature (Lond.)* 314: 366–369, 1985.
18. McCormack, J. E., Pepe, V. H., Kent, R. B., Dean, M., Marshak-Rothstein, A., and Sonenshein, G. E. Specific regulation of *c-myc* oncogene expression in a murine B-cell lymphoma. *Proc. Natl. Acad. Sci. USA* 81: 5546–5550, 1984.
19. Rabbitts, P. H., Watson, J. V., Lamond, A., Forster, A., Stinson, M. A., Evan, G., Fischer, W., Atherton, E., Sheppard, R., and Rabbitts, T. H. Metabolism of *c-myc* gene products: *c-myc* mRNA and protein expression in the cell cycle. *EMBO J.* 4: 2009–2015, 1985.
20. Spector, D. L. Macromolecular domains within the cell nucleus. *Annu. Rev. Cell Biol.* 9: 265–315, 1993.
21. Ascoli, C. A., and Maul, G. G. Identification of a novel nuclear domain. *J. Cell Biol.* 112: 785–795, 1991.
22. Carvalho, T., Seeler, J.-S., Ohman, K., Jordan, P., Pettersson, U., Akusjarvi, G., Carmo-Fonseca, M., and Dejean, A. Targeting of adenovirus E1A and E4-ORF3 proteins to nuclear matrix-associated PML bodies. *J. Cell Biol.* 131: 45–56, 1995.
23. Doucas, V., Ishov, A. M., Romo, A., Juguilon, H., Weitzman, M. D., Evans, R. M., and Maul, G. G. Adenovirus replication is coupled with the dynamic properties of the PML nuclear structure. *Genes Dev.* 10: 196–207, 1996.
24. Maul, G. G., Ishov, A. M., and Everett, R. D. Nuclear domain 10 as preexisting potential replication start sites of herpes simplex virus type-1. *Virology* 217: 67–75, 1996.
25. Chen, Y. M., Famer, A. A., Chen, C. F., Jones, D. C., Chen, P. L., and Lee, W. H. BRCA1 is a 220-kDa nuclear phosphoprotein that is expressed and phosphorylated in a cell cycle-dependent manner. *Cancer Res.* 56: 3168–3172, 1996.
26. Scully, R., Chen, J., Plug, A., Xiao, Y., Weaver, D., Feunteun, J., Ashley, T., and Livingston, D. M. Association of BRCA1 with Rad51 in mitotic and meiotic cells. *Cell* 88: 265–275, 1997.
27. Scully, R., Ganesan, S., Brown, M., De Caprio, J. A., Cannistra, S. A., Feunteun, J., Schnitt, S., and Livingston, D. M. Location of BRCA1 in human breast and ovarian cell lines. *Science (Washington DC)* 272: 123–125, 1996.
28. Tashiro, S., Kotomura, N., Shinohara, A., Tanaka, K., Ueda, K., and Kamada, N. S phase specific formation of the human Rad51 protein nuclear foci in lymphocytes. *Oncogene* 12: 2165–2170, 1996.

⁹ R. Wechsler-Reya and G. C. Prendergast, unpublished observations.

¹⁰ R. Wechsler-Reya, D. Sakamuro, J. Zhang, and G. C. Prendergast. Structural analysis of the human *BIN1* gene: evidence for alternate RNA splicing and tissue-specific regulation, submitted for publication.

BIN1 is a novel MYC-interacting protein with features of a tumour suppressor

Daitoku Sakamuro, Katherine J. Elliott, Robert Wechsler-Reya & George C. Prendergast

BIN1 is a novel protein that interacts with the functionally critical Myc box regions at the N terminus of the MYC oncoprotein. BIN1 is structurally related to amphiphysin, a breast cancer-associated autoimmune antigen, and RVS167, a negative regulator of the yeast cell cycle, suggesting roles in malignancy and cell cycle control. Consistent with this likelihood, BIN1 inhibited malignant cell transformation by MYC. Although *BIN1* is expressed in many normal cells, its levels were greatly reduced or undetectable in 14/27 carcinoma cell lines and 3/6 primary breast tumours. Deficits were functionally significant because ectopic expression of *BIN1* inhibited the growth of tumour cells lacking endogenous message. We conclude that BIN1 is an MYC-interacting protein with features of a tumour suppressor.

The MYC oncoprotein plays a central role in cell growth, apoptosis and malignancy (reviewed in refs 1–4). Following mitogenic stimulation of quiescent cells, MYC is rapidly induced and remains elevated, suggesting that it is needed for continuous cell growth. Its activation is sufficient to induce quiescent cells to enter the cell cycle⁵, while its inhibition can block mitogenic signals and drive cells toward terminal differentiation^{6–10}. MYC can also induce apoptosis, an event that occurs if its expression is uncoupled from the orchestration of other cell cycle regulatory events. For example, following growth factor withdrawal, cells that contain normal MYC downregulate its expression and exit the cell division cycle, while cells that contain deregulated MYC maintain its expression and undergo apoptosis^{11,12}. The molecular basis for these divergent biological effects is presently unknown.

A large body of evidence indicates that MYC can act as a transcription factor, with the C-terminal region involved in oligomerization and specific DNA recognition and the N-terminal region involved in transcriptional activation and repression (reviewed in refs 3, 4, 13). However, the extent to which strictly transcriptional activities account for all of MYC's biological activities is unclear. This issue might be clarified by elucidating the function of Myc boxes 1 and 2 (MB1 and MB2), two segments of evolutionarily conserved sequence whose integrity is critical for transformation and apoptosis^{12,14}. MB1 constitutes part of the N-terminal transcriptional activation domain (TAD) and contains cell cycle-regulated phosphorylation sites that may regulate TAD activity^{15–19}. MB2 has also been implicated in transactivation¹⁵ but a more important function may be

its role in transcriptional repression, which is mediated through promoter initiator (Inr) elements and is essential for transformation²⁰.

The Myc boxes, and MB1 in particular, are mutated in retroviral *Myc* genes and are hotspots for mutation in lymphomas and some carcinomas^{21–23}. Notably, the mutations found in lymphomas are frequently homozygous²². One interpretation of this finding is that the mutations cause a loss of MYC's ability to respond to negative regulators or to activate apoptotic pathways. Two studies have reported that some lymphoma mutations relieve inhibition of transactivation by p107 (refs 19, 24), a retinoblastoma (Rb)-related cell cycle regulator that can associate with the MYC N-terminal region^{24,25}. However, the significance of these results is uncertain as p107 has modest effects on MYC's transforming activity, is not a tumour suppressor, and has not been assigned any role in apoptosis. For these reasons, we have speculated that MYC may interact with other cellular polypeptides with greater significance for tumorigenesis. In this report we describe the identification of a Myc box-dependent binding protein, BIN1, which potently inhibits MYC transformation and has features of a tumour suppressor.

BIN1 is related to amphiphysin and RVS167

To identify proteins that interact with the N terminus of MYC, we focused on MB1 because it is the major hotspot for mutation in tumours and because, as the site of *in vivo* phosphorylation events¹⁸, it must be located on the polypeptide surface. To isolate candidate interacting proteins, a nontransactivating portion of MB1 was empirically chosen and used as 'bait' in a two hybrid screen (see Methods). Approximately 100 of several hundred

The Wistar
Institute, 3601
Spruce Street,
Philadelphia,
Pennsylvania
19104, USA

Correspondence
should be addressed
to G.C.P.
e-mail:
prendergast@
wista.wistar.
upenn.edu

a

```

MLWNVVTAGKIASNVQKLLTRAQEKVLQKLGKADETKDEQFEQCVQNFNK 50
QLTEGTRLQKDLRLTYLASVKAMHEASKKLNCEQLQEVYEPDWPGRDEANKI 100
AENNDLLWMDYHQKLVQALLTMDTYLGQFPDIKSRIAKRGRKLVVDYDSA 150
RHYESLQATKAKKDEAKIAKAEELIKAKQVFEMNVDLQELPSLWNSR 200
VGFYVNTFQSIAGLEENFHKMSKLNQNLNDVLVGLKQHGSSNTFTVKAQ 250
PRKSKLESRLRKNSNDNAPAGKNKSPSPDGSPAATPEIRVNHEPEPA 300
GGATPGATLPKSPSPQAEASEVAGGTQPAAGAQEPGETSASEAASSSLPA 350
S**S**AI*****V**A*****A*****T*****
VVVETFPATVNGTVEGGSGAGRLDLPPEMFKVOACHDYATATDTDELQK 400
*****S*****A**SA**T*****
AGDIVLVIPFONPEEODEGWLGVKESDMNOHKKLEKCRGVFPENFTERV* 451
*****

```

Fig. 1 BIN1 primary structure and relationship to amphiphysin and RVS167. **a**, Predicted primary sequence of human BIN1 from the 99f cDNA. Identities to the murine clone #99 ORF are depicted by asterisks; a six residue alignment gap is indicated by dashes. The SH3 motif is underlined and the NLS motif is underlined and italicized. It should be noted that ongoing genomic sequencing has revealed a difference between the N-terminal 5 residues of the 99f ORF shown here (MLWNV) and the ORF within a putative 5' exon of the human *BIN1* gene (MAEMGSKG). **b**, Structural relationship of BAR family proteins. Alignment of BIN1, human amphiphysin⁶³ and *S. cerevisiae* RVS167³³ was performed using the PILEUP algorithm of the Wisconsin Genetics package for the VAX computer and adjusted by visual inspection. Identities are boxed. Light shading demarcates the BAR domains and dark shading the SH3 domains; the unique BIN1 NLS is hatched; the lack of similarity between the BIN1 MBD and the central domains of amphiphysin and RVS167 are indicated by different hatching patterns in these regions. The amino acid residue positions that limit the extent of each region in BIN1, amphiphysin⁶³, and RVS167³³ are noted.

b

```

BIN1      MLWNVVTAGKIASNVQKLLTRAQEKVLQKLGKADETKDEQFEQCVQNFNKQLTEGTRLQKDLRLTYLASVKAMHEASKKLNCEQLQEVYEPDWPGRDEANKI 98
Amph1     ..NADIKTGLPANNVQKRLNRAQEKVLQKLGKADETKDEQFEYVQNFKPEAEETRLQKDLRLTYLASVKAMHEASKKLNCEQLQEVYEPDWPGRDEANKI 96
RVS167    .....MSFKGFTAAVSRNQSFQKFKMGQETQPEVYEDAEARRQELQETTKKISSESKRYSTANQMLTHQIGFAKSMHEITFKPISGKMSDPAAT 91

BIN1      .....KIAENNDLLWMDYHQKLVQALLTMDTYLGQFPDIKSRIAKRGRKLVVDYDSARHHYSELOARK.....KDEAKAKAEELIKAKQVFEMNVDLQELPSLWNSR 177
Amph1     .....VQKGVKLVWMDYHQKLVQALLTMDTYLGQFPDIKSRIAKRGRKLVVDYDSARHMLQALQSKR.....KDSIRZSKAEELIKAKQVFEMNVDLQELPSLWNSR 175
RVS167    TPEDNQGISAESEYRAIVAELOETAKPL.....ALVEEKVTPCQELKLTITTKMAKKNVKKULERHLNLTNNKHEKMSPTAKDSERLVKQAQVEV 189

BIN1      AOKVFEENVDLQELPSLWNSRVGFYVNTFQSIAGLEENFHKMSKLNQNLNDVLVGLKQHGSSNTFTVKAQPRKSKLESRLRKNSNDNAPAGKNKSPSPDGSPAATPEIRVNHEPEPA 266
Amph1     AOKVFEENVDLQELPSLWNSRVGFYVNTFQSIAGLEENFHKMSKLNQNLNDVLVGLKQHGSSNTFTVKAQPRKSKLESRLRKNSNDNAPAGKNKSPSPDGSPAATPEIRVNHEPEPA 275
RVS167    AQDEVDYNDILKLTQLHILFSLAEAEVKKLVNPFYPMOLNIFYTLNRLQKMKIPYFDLNSDIVESYIAKGNVEE.....QTDALITIFHKLGYSKA 282

BIN1      SLENTLAPASAPAPRPSPSQTKNGKPNVPLPKVTPTKELQENIISFFDNFVPEISVTPSQNEVPEVKKETLLDLDPDFKFPFVTPAGSNVTHSP 303
Amph1     SLENTLAPASAPAPRPSPSQTKNGKPNVPLPKVTPTKELQENIISFFDNFVPEISVTPSQNEVPEVKKETLLDLDPDFKFPFVTPAGSNVTHSP 375
RVS167    KLENTRRKYGVNAPAEVPSVGSASSG.....TGAGYDPAATATS..... 321

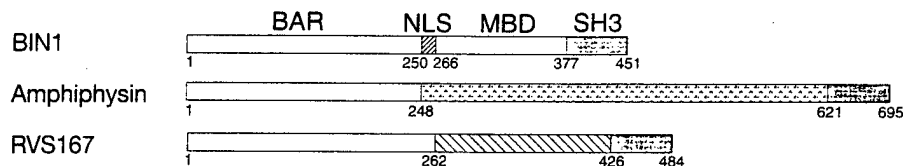
BIN1      .....TPGATLPKSPSQ.....PRASEV 322
Amph1     MSQTLPWDLWTTSDLVQPSAGSGSFNGTQPDSTLFTMTQDSMKLNLAESQAPPTPKAEPLAATVPAVLGLDGMDFRAEPEVVEAVIICADADA 475
RVS167    .....PTPTGYGYGAASFY.....AAQPAQYGTAAAVGTAA 360

BIN1      AESTOPAAEQE.PGE.....TSASDANSS.....LPAAVME.....TFPNTVNTMEZGSG 369
Amph1     AESTVSPAEGA.PGEEAAEAETVPAGEGVSLAEAKICQETTSASDANSS.....LPAAVME.....TFPNTVNTMEZGSG 574
RVS167    AESTVSPAEGA.PGEEAAEAETVPAGEGVSLAEAKICQETTSASDANSS.....LPAAVME.....TFPNTVNTMEZGSG 418

BIN1      AGR.....LPPFQPKVQACHDYATATDTDELQKAGDIVLVIPFONPEEODEGWLGVKESDMNOHKKLEKCRGVFPENFTERV* 429
Amph1     AGR.....LPPFQPKVQACHDYATATDTDELQKAGDIVLVIPFONPEEODEGWLGVKESDMNOHKKLEKCRGVFPENFTERV* 673
RVS167    AGR.....LPPFQPKVQACHDYATATDTDELQKAGDIVLVIPFONPEEODEGWLGVKESDMNOHKKLEKCRGVFPENFTERV* 468

BIN1      NHHKKLEKCRGVFPENFTERV* 451
Amph1     LQVRLDLYTKLFPENFTERV* 695
RVS167    .....QVFEENVDLQKLN 484

```



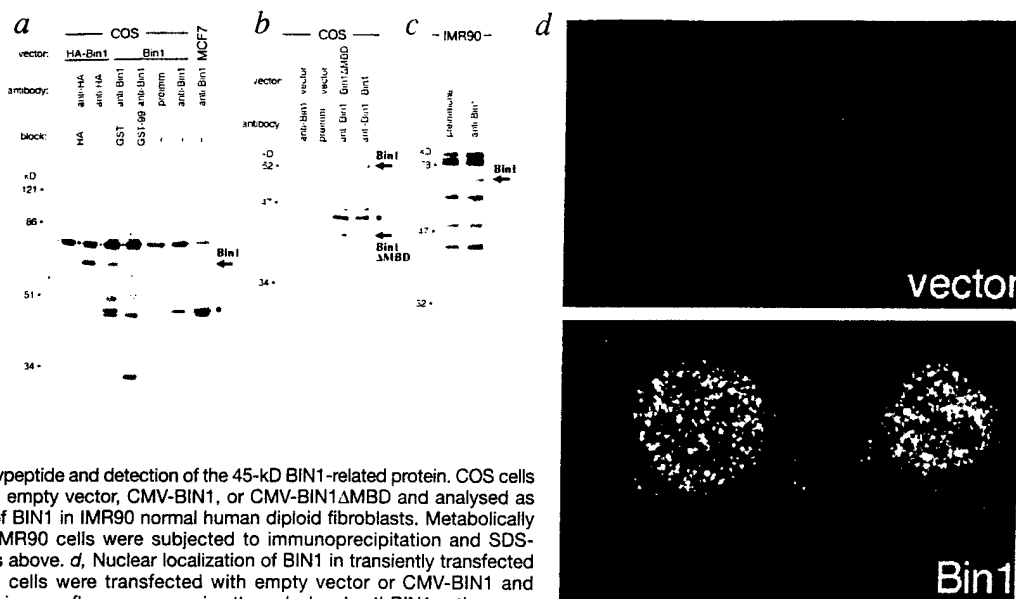
MB1-interacting candidates obtained in a screen of $\sim 3 \times 10^7$ cDNAs from a 10.5E murine embryo library were analysed by a mating strategy²⁶. Nonspecific interactions with empty vector, lamin, the small GTP-binding protein RhoB, or a protein kinase C (PKC) peptide were discarded. The PKC peptide contained a phosphothreonine site resembling the MB1 T58 site which is recognized by glycogen synthase kinase-3 (ref. 27), an enzyme present and active in yeast²⁸. This peptide was used to control for nonspecific peptide interactions (due to kinases, phosphatases, or peptidases). Analysis of 19 MB1-specific clones revealed that a single set of sequences was represented; one isolate, clone #99, was analysed in more detail. Clone #99 contained a 135-amino acid (aa) open reading frame (ORF) unrelated to known sequences. Preliminary results using clone #99 as a probe on northern blots indicated that a ~ 2.4 -kb RNA was specifically recognized in both human and murine cells.

Cloning and sequence analysis of a ~ 2.0 -kb human cDNA revealed a 451-aa ORF of predicted MW 50,049 that included a nuclear localization signal (NLS), a Src homology 3 (SH3) domain, and a central region of $\sim 89\%$ identity to clone #99; this central region was implicated as the MYC-interacting region (Fig. 1a). The predicted gene product was termed BIN1, for box-dependent myc-interacting protein-1. The near identity of murine and human BIN1 sequences strongly suggested that they were functional homologs. The presence of

the NLS suggested a nuclear location and function consistent with physiological MYC interaction; the presence of the SH3 domain, a protein-protein interaction interface found in many signal transduction proteins²⁹, suggested a role in signalling and interaction with other proteins. The SH3 domain was not involved in MYC interaction, as clone #99 lacked the complete domain and could still bind MYC (see below).

Comparison to the DNA database revealed extensive similarity in the terminal regions to two polypeptides, amphiphysin and RVS167 (Fig. 1b). Amphiphysin is a neuronal protein³⁰ that is the putative autoimmune antigen in breast cancer-associated Stiff-Man syndrome³¹, a paraneoplastic neurological condition³² seen in a small fraction of breast cancer patients. The meaning of this relationship was unclear but suggested connections of BIN1 to cancer. RVS167 is a negative regulator of the cell cycle in the yeast *S. cerevisiae*³³. This relationship suggested a role in cell cycle control and supported the notion of a negative regulatory or functional role for MB1. The high degree of structural similarity between the N-terminal regions of BIN1, amphiphysin, and RVS167 argued that they may share molecular function(s). For this reason, we have termed the N-terminal region of these proteins the BAR (Bin1/Amphiphysin/RVS167) domain. These BAR-containing proteins all contained C-terminal SH3 domains that were separated from the BAR domains by sequences unique to each protein (Fig. 1b).

Fig. 2 BIN1 protein expression and nuclear localization. **a**, Transient expression of a 70 kD polypeptide from *BIN1* cDNA. COS cells were transfected with CMV-BIN1 and subjected to immunoprecipitation 48 h later. Immunoprecipitates were analysed by SDS-PAGE and fluorography. The arrow indicates BIN1 and the dot a 45-kD BIN1-related polypeptide. **b**, Accumulation of the 42-kD BIN1 Δ MBD polypeptide and detection of the 45-kD BIN1-related protein. COS cells were transfected with empty vector, CMV-BIN1, or CMV-BIN1 Δ MBD and analysed as before. **c**, Detection of BIN1 in IMR90 normal human diploid fibroblasts. Metabolically labelled cultures of IMR90 cells were subjected to immunoprecipitation and SDS-PAGE/fluorography as above. **d**, Nuclear localization of BIN1 in transiently transfected HepG2 cells. HepG2 cells were transfected with empty vector or CMV-BIN1 and processed for indirect immunofluorescence using the polyclonal anti-BIN1 antisera.



BIN1 is a 70-kD nuclear polypeptide

A polyclonal antiserum was raised to a recombinant glutathione-S-transferase (GST) fusion protein which included the unique central region of BIN1 overlapping with the clone #99 ORF (the terminal regions were omitted from the immunogen to reduce possible crossreaction with other BAR family proteins). When incubated with metabolically labelled extracts from COS cells transiently transfected with a BIN1 expression vector (CMV-BIN1), this antisera immunoprecipitated two polypeptides with apparent MW of 70 kD and 45 kD (Fig. 2a). Both polypeptides were specifically recognized since their appearance was blocked by preincubating antisera with a molar excess of the GST-BIN1 immunogen but not with unfused GST. By several criteria we established that the 70-kD species was BIN1 while the 45-kD species represented a BIN1-related polypeptide. First, in COS cells transiently transfected with a viral haemagglutinin (HA) epitope-tagged BIN1 construct, only the 70-kD polypeptide was recognized by an anti-HA monoclonal antibody (Fig. 2a). Second, the *in vitro* translation (IVT) product from the full-length cDNA also displayed a mobility of 70 kD (data not shown). Third, only the 45-kD polypeptide was detected in untransfected MCF7 breast tumour cells (Fig. 2a), which lacked *BIN1* RNA (see below), or in cells transfected with empty vector (Fig. 2b). This proved that the 45-kD species was not a coprecipitant or a processed or degraded form of BIN1. Consistent with its assignment as a BIN1-related protein, the 45-kD species was detected by western blotting in several human cell lines (data not shown). We observed stable accumulation of a polypeptide with a predicted and apparent MW of 42 kD following COS transfection with CMV-BIN1 Δ MBD (Fig. 2b), a deletion construct lacking the central region implicated as the MYC-binding domain (MBD, aa 270–377; see below). This result indicated that full-length BIN1 migrated aberrantly at 70 kD, instead of at the predicted MW of 50 kD, because of an MBD determinant.

To establish that the *BIN1* cDNA encoded a polypeptide similar to that found in normal cells, metabolically

labelled extracts from IMR90 human diploid fibroblasts were subjected to immunoprecipitation analysis. A similar 70 kD polypeptide was specifically recognized by immune but not preimmune sera (Fig. 2c). The cellular localization of BIN1 was examined by indirect immunofluorescence of transiently transfected cultures of HepG2 hepatocarcinoma cells, which, like MCF7 cells, lack detectable *BIN1* RNA (see below) and therefore provided an internal control for any crossreacting polypeptides. HepG2 cells transfected with CMV-BIN1 exhibited a bright nuclear staining pattern which was absent from cells transfected with empty vector (Fig. 2d). The nuclear

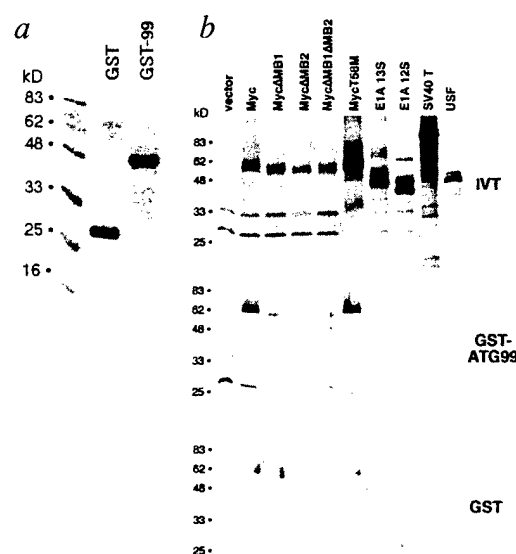


Fig. 3 Myc box-dependent interaction. **a**, GST-99 expression. A GST-clone #99 fusion protein was purified from recombinant *E. coli* and examined by SDS-PAGE and Coomassie staining. **b**, Selective biochemical interaction between MYC and GST-99. Binding assays were performed with equivalent quantities of GST or GST-99 and the indicated *in vitro* translated polypeptides. The bound fraction was analysed by SDS-PAGE and fluorography. IVT, one-tenth volume of *in vitro* translated polypeptide added to each binding reaction was examined.

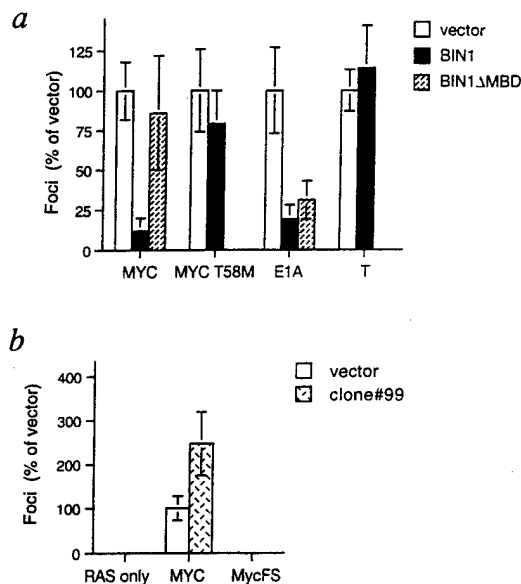


Fig. 4 *BIN1* inhibits *MYC* transformation in a manner requiring the integrity of *MYC* MB1 and the presence of the *BIN1* MBD. **a**, Selective requirement of the MBD for *MYC* inhibition. REFs were transfected with a 2:1:1 ratio of empty vector, CMV-BIN1, or CMV-BIN1ΔMBD, activated RAS, and MYC, E1A, or SV40 T antigen expression vectors and transformed foci were scored two weeks later. The data represent three to seven trials for each transfection. The data are depicted as the percent of foci induced by oncogenes + vector, as appropriate. **b**, Dominant inhibitory activity of MBD. REFs were transfected as above except with a 2:1:1 ratio of empty vector of CMV-ATG99, a clone #99 expression vector, to oncogene plasmids. In this experiment, a *MYC* frameshift mutant, *MYC-FS*, was included to establish that the augmentation of foci formation by CMV-ATG99 was *MYC*-dependent.

localization was consistent with the presence of a NLS in the *BIN1* sequence and with a nuclear site of *MYC* interaction. We conclude that the *BIN1* cDNA encodes a nuclear protein with an apparent MW of ~70 kD, identical to a protein found in normal human fibroblasts.

Biochemical association between *BIN1* and *MYC*

To confirm a biochemical interaction with *MYC*, we expressed the clone #99 ORF in *E. coli* as a GST fusion protein (GST-99; Fig. 3a) and tested for binding to [³⁵S]-methionine-labelled *MYC* or control polypeptides synthesized by IVT. While unfused GST did not bind *MYC*, the GST-99 fusion was able to bind *MYC* in a specific manner (Fig. 3b). Although only the MB1 portion of *MYC* was used as 'bait' in the two hybrid screen, binding of GST-99 required both the MB1 and MB2 portions of the full-length oncoprotein, since deletion of either box region significantly reduced *MYC* binding. We interpreted this to mean either that MB1 was occluded in the MB2 deletion mutant or that MB1 was sufficient for interaction in yeast because of overexpression or other conditions which facilitated binding (for example, particular bait fusions). In the binding assays, approximately 10% of the input *MYC* protein bound to GST-99. This was not due to weak avidity because, once formed, the *MYC*-GST-99 complex was stable in buffers containing up to 500 mM NaCl. Binding was also observed with a *MYC* MB1 mutant, *MYC* T58M, which has lost the T58 negative regulatory phosphorylation site. GST-99 did not interact detectably with other nuclear transforming proteins, such as the SV40 T antigen or the

adenovirus E1A gene products, nor did it bind the *MYC*-related basic/helix-loop-helix/'leucine zipper' (b/HLH/Z) protein upstream stimulatory factor (USF). In addition, the other *MYC*-interacting polypeptides MAX, p107, or YY1 did not associate with GST-99 (data not shown). We conclude that *BIN1* directly and specifically associates with *MYC* in a manner that depends upon the critical *MyC* box regions.

BIN1 inhibits *MYC* transformation

The ability of *BIN1* to associate with *MYC* depended on the presence of MB1 and MB2, which are required for transformation activity^{14,20,27}. Therefore, we tested the effects of *BIN1* and *BIN1*ΔMBD on *MYC* transformation in the RAS cooperation assay³⁴ performed in primary rat embryo fibroblasts (REFs). For specificity controls, additional experiments were performed in which *MYC* was replaced by either adenovirus E1A or SV40 T antigen, which can also cooperate with RAS in this assay. As *MYC* mutants that cannot be phosphorylated at the MB1 T58 residue have been reported to escape p107-mediated inhibition of transactivation¹⁹, we also asked whether the *MYC* T58M mutant could escape any effects of *BIN1*. Finally, as the original clone #99 ORF was partial and encoded essentially only the MBD, we anticipated that it might act in a dominant negative manner. Therefore, the effects of a clone #99 expression vector on *MYC* transformation were also examined.

The *BIN1*-containing vectors, alone or with activated RAS, lacked transforming activity. However, when cotransfected at a 2:1:1 ratio with *MYC* and RAS, *BIN1* selectively inhibited focus formation ~7-fold (Fig. 4a). Inhibition could be titrated by decreasing the ratio of *BIN1* to *MYC* and RAS in the transfected DNA (data not shown). Moreover, inhibition was dependent on *MYC* binding, since *BIN1*ΔMBD, which lacks the *MYC*-binding domain, was inactive in this assay. The loss of inhibition could not be attributed to protein instability, since *BIN1*ΔMBD was shown to stably accumulate in transfected cells (Fig. 2b), and could inhibit transformation by E1A (see below). In contrast to its effects on wild-type *MYC*, *BIN1* did not efficiently inhibit transformation by *MYC* T58M, even though this mutation did not affect binding (Fig. 3b). Thus, mutation of the MB1 T58 residue or deletion of the *BIN1* MBD relieved *BIN1* inhibition of *MYC* transformation.

We anticipated that E1A would be inhibited by *BIN1* since E1A acts similarly to *MYC* in focus formation and apoptosis assays^{12,34-36}. Confirming expectations, *BIN1* inhibited cell transformation by E1A. In contrast, cell transformation by T antigen was unaffected. This result argued that the effects of *BIN1* on *MYC* and E1A were not due to toxicity or to nonspecific inhibition of the transformed phenotype. Notably, while *BIN1*ΔMBD lacked the ability to inhibit *MYC*, it could still suppress E1A transformation. This suggested that *BIN1* inhibited *MYC* and E1A by different mechanisms. We could not rule out the possibility that intrinsic differences in the sensitivity of E1A and *MYC* to *BIN1* inhibition were due to a reduced activity of *BIN1*ΔMBD. However, the difference in susceptibility to *BIN1*ΔMBD was consistent with the observation that *MYC*, but not E1A, could associate with the *BIN1* MBD region (Fig. 3b).

When a clone #99 vector was introduced into REFs instead of *BIN1*, an effect opposite to that of the full-

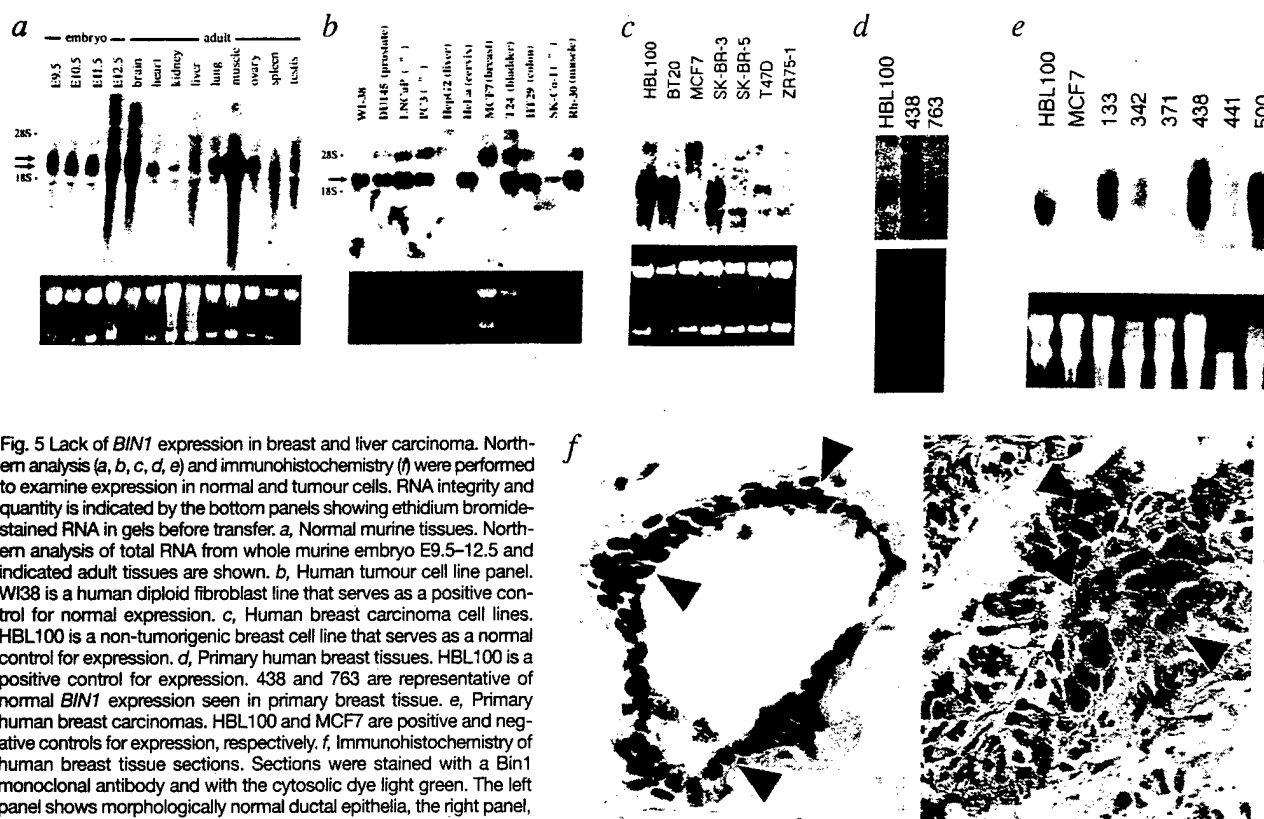


Fig. 5 Lack of *BIN1* expression in breast and liver carcinoma. Northern analysis (a, b, c, d, e) and immunohistochemistry (f) were performed to examine expression in normal and tumour cells. RNA integrity and quantity is indicated by the bottom panels showing ethidium bromide-stained RNA in gels before transfer. a, Normal murine tissues. Northern analysis of total RNA from whole murine embryo E9.5–12.5 and indicated adult tissues are shown. b, Human tumour cell line panel. WI-38 is a human diploid fibroblast line that serves as a positive control for normal expression. c, Human breast carcinoma cell lines. HBL100 is a non-tumorigenic breast cell line that serves as a normal control for expression. d, Primary human breast tissues. HBL100 is a positive control for expression. 438 and 763 are representative of normal *BIN1* expression seen in primary breast tissue. e, Primary human breast carcinomas. HBL100 and MCF7 are positive and negative controls for expression, respectively. f, Immunohistochemistry of human breast tissue sections. Sections were stained with a *Bin1* monoclonal antibody and with the cytosolic dye light green. The left panel shows morphologically normal ductal epithelia, the right panel, a portion of a tumour mass. Arrowheads indicate cell nuclei.

length protein was observed. Cotransfection of clone #99 with *MYC* and *RAS* led to a ~2.4-fold augmentation of *MYC* transformation activity (Fig. 4b). This result suggested that clone #99 acted to dominantly inhibit an endogenous REF *Bin1* protein. Confirming a dominant inhibitory effect, we observed that titration of clone #99 into the REF assay could reverse inhibition of *MYC* and *RAS* by *BIN1* (data not shown).

The *in vitro* biochemical association results, together with the REF transformation data, provided genetic evidence that *BIN1* and *MYC* could interact *in vivo*. First, mutations in either molecule (T58M in MB1, MBD in *BIN1*) eliminated *BIN1* inhibition. Second, a portion of *BIN1* encompassing the MBD alone (clone #99) increased *MYC* transforming activity through a dominant inhibitory activity. Finally, since the MBD was sufficient and the *Myc* boxes were necessary for association *in vitro* (Fig. 3b), there was good correlation between the regions involved in protein–protein association and the regions required for biological action. We conclude that *BIN1* inhibits *MYC* by directly interacting with it *in vivo*.

***BIN1* is absent in tumour cells**

BIN1 was hypothesized to be a tumour suppressor gene product, because it inhibited transformation by *MYC* through interaction with a region that is mutated in tumours. Since a hallmark of tumour suppressors is loss of function in tumour cells (due to genetic or epigenetic causes), we performed northern analyses of RNA from murine and human cells. We found that *BIN1* message was ubiquitous in normal cells. In murine tissues, levels were highest in embryo, adult brain and adult muscle,

with lower levels seen in all other tissues examined (Fig. 5a). At least two transcripts could be seen in embryo and brain, suggesting alternate splicing or differentially regulated sites of RNA initiation or polyadenylation in these tissues. In human cells, similar levels of *BIN1* RNA were seen in WI-38 normal diploid fibroblasts and tumour cell lines derived from various tissues, with the notable exception of HepG2, a hepatocarcinoma cell line, and MCF7, a breast carcinoma cell line, where no message was detected (Fig. 5b).

Further analysis of breast carcinoma cells indicated that *BIN1* expression was frequently missing. *BIN1* RNA was absent or reduced in 5/6 breast carcinoma cell lines examined (Fig. 5c). The absence did not reflect the integrity of the RNA, nor the general lack of expression of *BIN1* in these breast cells, because message was plainly detectable in HBL100, a flat non-tumorigenic breast cell line (Fig. 5c), as well as in a panel of RNAs isolated from primary breast tissues (Fig. 5d). Similar deficits were also seen in 3/6 cervix, 1/2 lung, and 3/6 other liver tumour cell lines (data not shown), suggesting that lack of *BIN1* expression may be common to many carcinomas. We also observed a lack of *BIN1* message in RNA isolated from 3/6 primary breast tumours (Fig. 5e). These data indicated that lack of expression was not a feature of tumour cell line establishment or long-term culture *in vitro*. Finally, we corroborated the data from primary tumours by immunohistochemical analysis of breast tissue sections, using a *BIN1*-specific monoclonal antibody (R.W.-R., D.S., M. Herlyn and G.C.P., unpublished results; an example of these data is shown in Fig. 5f). Cells were counterstained with the cytoplasmic dye light green. *BIN1* staining was present in the cell nuclei

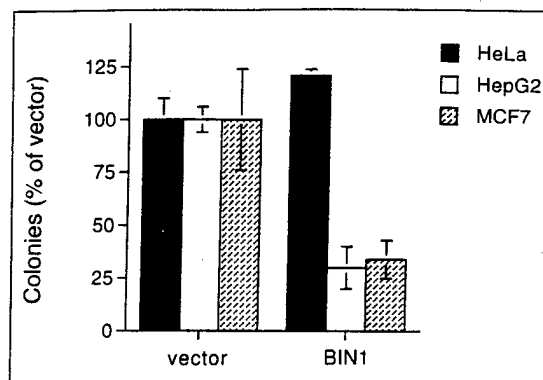


Fig. 6 *BIN1* selectively inhibits colony formation in cells lacking endogenous expression. HeLa, HepG2, and MCF7 cells were transfected with the plasmid CMV-*BIN1* or with empty vector, passaged 1:4 into three dishes each, and selected for the neomycin gene present on each plasmid by culturing in G418. Drug-resistant colonies were scored 2–3 weeks later. The data are depicted as the percentage of colonies obtained with empty vector.

of morphologically normal ductal epithelia (Fig. 5f, left panel). The pattern seen was consistent with the nuclear localization seen earlier (Fig. 2d) and was specific since incubation of sections with secondary antibody alone produced no staining (data not shown). Consistent with the results from northern analysis, there was little or no staining of frank carcinoma cells (Fig. 5f, right panel). We concluded that *BIN1* expression may be frequently missing in carcinoma of breast and other tissues.

BIN1 inhibits tumour cell growth

To assess the significance of the *BIN1* deficits seen in carcinoma cells, we determined the effect of ectopic *BIN1* expression in tumour cells lacking endogenous *BIN1*. This was done by performing G418-resistant cell colony formation experiments with cell lines shown to contain (HeLa) or lack (HepG2, MCF7) *BIN1* message. In the latter cell lines, a *BIN1* vector carrying a neomycin-resistance gene exhibited a ~3-fold reduction in colony formation efficiency relative to empty vector alone (Fig. 6). This could not be explained by either a general toxic effect or reduced transfection efficiency because the colony formation efficiency of both vectors was similar in HeLa cells. When examined by immunoprecipitation, cell populations derived from pooled colonies which emerged from *BIN1*-transfected HepG2 cultures showed no evidence of expression (data not shown), consistent with an incompatibility with cell growth. We concluded that the expression deficits seen in carcinoma cells were functionally significant and that *BIN1* was capable of exerting a tumour suppressor activity.

Discussion

We have identified *BIN1* as a novel protein that specifically interacts with MYC and inhibits its oncogenic activity. Cell transformation experiments using MYC and *BIN1* mutants provided genetic evidence of *in vivo* interaction. The primary structure of *BIN1* suggested a negative role in cell cycle control and hinted at connections to breast cancer. Consistent with a role as a tumour suppressor, *BIN1* expression was found to be ubiquitous in normal cells but frequently missing in carcinoma cells, where the deficits were demonstrated to be functionally significant.

The structural similarity of *BIN1* to amphiphysin is

provocative given the compelling causal links between MYC deregulation and breast carcinoma^{37–42}. Although the meaning of the amphiphysin relationship is unclear, it prompts consideration of the immunological as well as the functional aspects of *BIN1* in breast cancer. The relatedness of *BIN1* to RVS167 suggests a function in cell cycle control. RVS167 is a nonessential gene product that is required for nutrient-deprived yeast cells to exit the cell cycle³³. Consistent with the structural relationship, *BIN1* appeared to be dispensable for growth, while enforced expression could suppress MYC transformation and tumour cell proliferation. Despite this parallel, however, *BIN1* was unable to complement an RVS167 null mutation (G.C.P., unpublished data), indicating that these proteins are structurally related but are not functionally homologous. The difference may be due to the presence of divergent sequences in the central region of each protein, where the *BIN1* MBD is found. Nevertheless, it is reasonable to search for clues to *BIN1* function among the features shared with other BAR family members. The most obvious one is the presence of a SH3 domain in the C terminus. SH3 domains are typically observed in signal transduction proteins which control or are affected by changes in cell structure²⁹. Indeed, RVS167 has been implicated in actin cytoskeletal control and budding³³ and amphiphysin may have a role in synaptic vesicle endocytosis⁴³. By analogy, one would predict that *BIN1* has a role in signal transduction linked to some aspect of cell structure control, presumably in the nucleus.

The basis for *BIN1* activity is not yet clear. *BIN1* structure lacks obvious features of a transcription factor or transcriptional adaptor protein. However, the role of the Myc boxes in transcription^{15,20} and the ability of MYC T58M to escape inhibition by *BIN1*, suggests that *BIN1* may act by affecting transcription. We observed that T58 mutation did not affect *BIN1* binding, but could relieve inhibition of MYC transformation. Others have reported that T58 mutation does not affect p107 binding but can relieve p107 inhibition of MYC transactivation^{19,24}. Consistent with the possibility that it may act at least in part by blocking transactivation, data from preliminary experiments indicate that *BIN1* can inhibit MYC-dependent transactivation of α -prothymosin (S. Gaubatz, K.E., D.S., G.C.P. and M. Eilers, unpublished results), ornithine decarboxylase (D.S. and G.C.P., unpublished results) and CDC2 (T. Born, K.E., D.S., G.C.P. and J. Feramisco, unpublished results), three MYC-regulated genes^{44,45,46}. The means by which *BIN1* acts appears to be different, though, because unlike p107, *BIN1* does not appear to affect transactivation from minimal promoters containing multimerized MYC binding sites (K.E. and G.C.P., unpublished data). In future work it will be important to determine whether this effect of *BIN1* represents an active inhibitory role or simply reflects a passive effect of MYC binding, due to competition for functional association with *bona fide* transcriptional adaptor proteins.

Our results are consistent with a role for *BIN1* in MYC regulation, like that proposed for p107^{24,25}, but they are also consistent with a role in MYC-mediated apoptosis^{11,12}. We have previously shown that MYC can induce apoptosis in epithelial cells by p53-independent mechanisms⁴⁷ and the frequent loss of *BIN1* in carcinomas is intriguing in this regard. However, regardless of how it

acts, our results support the notion that BIN1 interacts with MYC *in vivo* to some functional end. One caveat to this interpretation is that coimmunoprecipitation experiments performed in an effort to identify a cellular complex containing BIN1 and MYC have yielded largely negative results to date. Some evidence of specific association has been obtained from extracts from Sf9 insect cells coinfecting with recombinant baculoviruses. However, even under such conditions of overexpression, only a small fraction of BIN1 has been seen to remain associated with MYC following immunoprecipitation (K.J.E., unpublished results). Given that the BIN1 interaction may be regulatory in nature, a complex between full-length proteins may be inherently unstable, transient, or require unidentified cofactors or extraction conditions to remain stably associated following cell lysis. Moreover, given the difficulties widely experienced with MYC biochemical analyses, the question of *in vivo* interaction might be best addressed by using a genetic approach based on transcription experiments. In future work, such experiments could also be used to test whether Myc box mutations seen in tumour cell MYC genes can defeat BIN1 inhibition and whether MYC mutation might be functionally equivalent, in some cases, to loss of BIN1 expression.

Several lines of evidence support the conclusion that BIN1 represents a tumour suppressor function. First, BIN1 selectively inhibits cell transformation by MYC, which is involved in many human cancers. Second, BIN1 shows structural similarity to RVS167, a negative regulator of the yeast cell cycle which is dispensable for proliferation. Third, BIN1 is ubiquitously expressed in normal tissues but frequently missing in carcinoma cells. Fourth, BIN1 selectively inhibits the growth of carcinoma cells lacking endogenous expression. These results show that the observed expression deficits are functionally significant, rather than simply correlated with loss of genomic integrity, and formally demonstrate that BIN1 can act as a tumour suppressor. Finally, the human BIN1 gene has been mapped to chromosome 2q14 (ref. 48), a locus lying within a mid-2q region deleted in 50% of metastatic prostate carcinomas⁴⁹ and, at the syntenic murine locus, in 90% of radiation-induced myeloid leukaemias⁵⁰. In future work, it will be important to determine whether lack of BIN1 expression, where it occurs, is due to genetic or epigenetic causes.

Note added in proof: The murine homologue of BIN1 was cloned recently in an expression screen for novel SH3 domain-containing proteins (Sparks, A.B., Hoffman, N.G., McConnell, S.J., Fowlkes, D.M. & Kay, B.K. *Nature Biotech.* 14, 741–744, 1996).

Methods

Two hybrid assay. A two hybrid system²⁶ and a murine embryo E10.5 cDNA library (a gift from S. Hollenberg) were used to identify clones that interacted with the MB1 peptide EDIWKKEELLPTPLS (human MYC aa 47–62). The bait plasmids were based on pBTM116 (P. Bartel and S. Fields, unpublished results), which uses *lexA* as a DNA binding component and contains a TRP1 marker. The library vector, pVP16, uses a region of herpes simplex virus VP16 as a transcriptional transactivating component and contains a LEU2 marker. cDNA synthesized from 10.5d murine embryo RNA was size-selected by random DNase I treatment to ~0.5 kb, treated with Klenow enzyme, *NotI* linker, and subcloned into pVP16 (S. Hollenberg, unpublished data). This cDNA library was designed to express protein

modules whose interactions might be occluded in full-length polypeptides. The yeast strain L40 (*MATa trp1-901 leu2-3,112 LYS2::(lexAop)₄-HIS3 URA3::(lexAop)₈-lacZ*) was used in the assay. Approximately 3×10^7 TRP⁺LEU⁺ transformants were examined in the primary screen, 200–300 of which were also HIS⁺LacZ⁺. One hundred clones cured of the bait plasmid were tested for interaction by a mating strategy using AMR70 (R. Sternglanz, unpublished data). Test baits in AMR70 included the original *lexA*-MB1 construct and a set of negative controls including no insert, *lamin*²⁶, the small GTP-binding protein RhoB³¹, or the peptide FTRHPPVLTTPDQEV1 derived from rat protein kinase C β 2, which contains a threonine phosphorylation site analogous to MB1 residue T58 (G.C.P. and K. Koblan, unpublished results).

Biochemical association assay. The ~0.5-kb murine clone #99 cDNA insert on a *Clal*-*EcoRI* fragment was substituted for a similar fragment in pE47 (ref. 52), making pATG-99. The pATG-99 ORF included an initiator methionine, added a 15-aa N-terminal extension (3 aa from E47 and 12 aa from VP16) to the 135 residue clone #99 ORF, and retained the translational termination site derived from the two hybrid vector. Expression of the ATG99 polypeptide was confirmed by *in vitro* translation from pATG-99. The pATG-99 insert was subcloned into pGEX-2T (Pharmacia) and GST-99 polypeptide was expressed and purified from *E. coli* cell extracts on glutathione-Sepharose (Pharmacia), using protocols supplied by the vendor. [³⁵S]-methionine labelled polypeptides were generated by IVT using TNT rabbit reticulocyte lysates (Promega). Expression plasmids included the c-Myc expression plasmid CMV Hm³³; CMV Hm subclones containing the MB1 deletion aa 49–101 (ref. 14), MB2 deletion aa 120–140 (ref. 20), or both deletions; the adenovirus E1A expression plasmids p12S, p13S; the SV40 T antigen expression plasmid (provided by F. Rauscher); and CMV-USF²⁰. Approximately 2.5 µg of GST or GST-99 and 10 µl of an IVT reaction were added to 0.5 ml binding buffer (10 mM Tris-HCl pH 7.5, 5 mM EDTA, 500 mM NaCl, 0.25% NP40), incubated 1 h at 4°C on a nutator shaker, washed four times with binding buffer, and analysed by SDS-PAGE and fluorography.

cDNA cloning and characterization. Using standard methods, a human skeletal muscle λ ZAPII cDNA library (Stratagene) was hybridized with [³²P]-labelled clone #99 insert and washed under low stringency conditions (2× SSC, 42°C). The complete DNA sequence of a subcloned ~2.0-kb cDNA, p99f, was determined using the dideoxy method with Sequenase (US Biochemicals) and assembled and analysed with MacVector software (IBI/Kodak). DNA database comparisons were performed using BLAST client software.

Expression vectors. BIN1 expression vectors were constructed as follows. CMV-BIN1 was generated by subcloning a 1.6-kb *EcoRI* fragment from p99f that contained its entire predicted coding sequence into pcDNA3 (Invitrogen), a mammalian cell expression vector that contains a cytomegalovirus enhancer/promoter and a 3' polyadenylation signal. BIN1 CMV-HA was constructed by substituting a *PvuII*-*EcoRI* coding region fragment from CMV-BIN1 for an *EcoRV*-*EcoRI* fragment of neoCMV-hem rhoA (G.C.P., unpublished results), a RhoA expression plasmid that included an 8 residue N-terminal viral haemagglutinin (HA) epitope recognized by the monoclonal antibody 12CA5 (ref. 52). The HA-Bin1 polypeptide created included residues 1–47 from the N terminus of HA-RhoA and residues 52–451 of Bin1 in CMV-HA-Bin1, adding an N-terminal extension that proved necessary for immunoprecipitation by anti-HA antibody 12CA5 (ref. 54). CMV-BIN1 Δ MBD deleted aa residues 270–377 in CMV-BIN1. It was generated by ligating two separate PCR fragments generated by the 5' primer CCGGATCCGCGATGCTCTGGAACGTGGTGACG and the 3' primer GCGAATTCGTTGTCACTGTTCTTCTTCTGCG (fragment encoding 1–269) and the 5' primer CGGAATTCACCATGGGTTTCATGTTCAAGGTACAG and the 3' primer CCGCTCGAGTCATGGGAC-

CTCTCAGTGAAGTT (fragment encoding 378–451). This construction included the nonspecific amino acids EFTM at the fusion junction due to the restriction site added. CMV-ATG99 was generated by subcloning the insert from pATG-99 into pcDNA3.

LTR Hm, which contains a Moloney long terminal repeat-driven normal human *MYC* gene, and pT22, which contains an activated Ha-RAS gene, have been described^{34,55}. LTR Hm T58M was generated by subcloning in LTR Hm a fragment containing the T58M mutation from the less potently transforming vector CMV Hm T58M⁴⁵. A nontransforming *MYC* frameshift mutant (MycFS) was constructed by digestion of LTR Hm with a unique *Bst*II in exon 2 of the human *MYC* gene, filling with Klenow enzyme, and self ligation. The MycFS polypeptide encoded by this mutant, LTR Hm/Bst, is frameshifted at aa residue 104 (G.P., unpublished data). neoCMV T and p1A/neo, encoding SV40 T antigen and adenovirus E1A, were obtained from N. Kohl.

Tissue culture. Human COS, HeLa, HepG2, MCF7, and IMR90 cells were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (Sigma) and 50 U/ml each penicillin and streptomycin (Fisher). Primary rat embryo fibroblasts (REFs; Whittaker Bioproducts) were cultured and transfected as described⁵⁶. Briefly, secondary passage REFs seeded into 10 cm dishes were transfected overnight by a calcium phosphate coprecipitation method⁵⁷ with 5 µg each of oncogene plasmids and 10 µg of other plasmids indicated, then passaged 1:5 the next day and fed with normal growth media until foci were scored by methanol fixation and crystal violet staining 12–14 d later. In some experiments, 0.5 mg/ml G418 was added the day after passaging to address the possibility that *BIN1* inhibited cell growth as well as focus formation. Colony formation assays in HeLa, HepG2, and MCF7 were performed by seeding $\sim 3 \times 10^5$ cells in 3 cm dishes and transfecting the next day with 2 µg CMV-BIN1 or vector using Lipofectamine (Gibco/BRL). Cells were passaged 48 hr after transfection at a 1:10 ratio into 6 cm dishes containing media with ~ 0.6 mg/ml G418. Cell colonies were scored by crystal violet staining 2–3 wk later.

Immunoprecipitation. Two BIN1 antibodies were used in this study. A polyclonal antiserum used in the protein characterization experiments (Fig. 2) was generated by immunizing rabbits with a GST fusion protein containing aa residues 189–398 of BIN1 (GST-99Pst) through a commercial service (Rockland, Inc., Boyerstown PA). A BIN1-specific monoclonal antibody, 99D, was used in the coimmunoprecipitation and immunohistochemical experiments (Figs 3c, 5g). 99D was raised to the same immunogen as used for the polyclonal antiserum and is specific for the ~ 70 kD BIN1 polypeptide (R.W.-R., D.S., M. Herlyn and G.C.P., unpublished data).

IMR90 or transiently transfected COS cells were metabolically labelled for 2–4 h in DMEM media lacking methionine and cysteine (Gibco) with 75–125 µCi/ml EXPRESS labelling reagent (NEN) and cell extracts were prepared with RIPA buffer containing the protease inhibitors leupeptin, aprotinin, phenylmethylsulfonyl fluoride, and antipain⁵⁸. Extracts were centrifuged at 20000g for 15 min at 4°C before use. Before the addition of BIN1 antibodies, extracts were precleared by a 1 h treatment with prebleed sera or normal mouse IgG and 20 µl of a 1:1 slurry of protein G Sepharose beads at 4°C on a nutator (Pharma-

cia). Immunoprecipitation proceeded at least 90 min at 4°C followed by the addition of protein G beads and a further 30 min incubation. Beads were collected by brief centrifugation, washed four times with buffer, boiled in SDS gel loading buffer, fractionated on 10% gels, and fluorographed.

Immunofluorescence. HepG2 cells ($\sim 5 \times 10^3$) were seeded onto glass cover slips in 6 cm dishes and the next day transfected overnight with 4 µg CMV-BIN1 or pcDNA3. Two days later cells were washed and processed for immunofluorescence essentially as described⁵⁹, using 5 µg of protein A Sepharose-purified anti-BIN1 IgG and a 1:1000 dilution of fluorescein-conjugated anti-rabbit IgG (Cappel) as the secondary antibody. Stained cover slips were digitally analysed on a Leitz confocal microscope.

Immunohistochemistry. Frozen human breast tumour tissues were a gift from D. Herlyn. Tissue sections on cover slips were prepared for staining by fixing 30 min at 4°C with 4% paraformaldehyde and permeabilizing by treatment 3 min with 0.1% Triton X-100. Endogenous peroxidase was quenched by incubating slips 20 min in 0.3% methanol. Tissue was blocked 20 min with 10% normal goat serum in PBS/0.1% BSA, washed, and incubated 30 min in the same buffer with 1:10 dilution of hybridoma supernatant. The BIN1 staining pattern was identified by incubation 30 min with a goat horseradish peroxidase-conjugated anti-mouse antibody (Jackson ImmunoResearch) followed by a 5 min incubation with substrate. Before mounting, slides were counterstained with by a 1 min incubation with 0.04% acidified solution of the cytoplasm dye light green. Stained sections were photographed at $\times 500$ magnification.

Northern analysis. Total cytoplasmic RNA was isolated as described⁶⁰ from human WI-38 cells and tumour cell lines. Total RNA was isolated as described⁶¹ from frozen normal and tumour breast tissues obtained from the Cooperative Human Tissue Network (Philadelphia, PA). Total RNA from murine embryo and adult tissues was a gift from L. Benjamin. RNAs were fractionated on formaldehyde gels and hybridized to [³²P]-labelled *BIN1* cDNA probes⁶².

Acknowledgements

This manuscript is dedicated to the memory of Robert Hopewell. We thank S. Hollenberg for the two hybrid system and module cDNA library; L. Benjamin, M. Cole, M. Crouzet, P. Durrens, C. Howe, K. Koblan, N. Kohl, F. Rauscher, D. Simon and B. Weber for reagents; D. Ewert and W. Olsen for help with immunohistochemistry; D. Herlyn for frozen embedded breast tissues; M. Herlyn for monoclonal antibodies; D. Marchadier for recombinant baculoviruses; R. Buccafusca, T. Britten, C. Wright and the Wistar Microscopy, Expression Vector, Monoclonal Antibody, and Histology Core Facilities for technical help; and S. Berger, T. Halazonetis, F. Rauscher, G. Rovera and D. Simon for critical comments and discussion. This work was supported in part by NIH grants CA10815 and CA66179, and a grant from Merck and Co., Inc. K.J.E. was supported by NIH Training Grant CA09171. R.W.-R. is an MRC Canada Postdoctoral Fellow. G.C.P. is the recipient of an American Cancer Society Junior Faculty Award and is a Pew Scholar in the Biomedical Sciences.

Received 2 May; accepted 14 June 1996.

1. Cole, M.D. The myc oncogene: Its role in transformation and differentiation. *Annu. Rev. Genet.* **20**, 361-384 (1986).
2. Evan, G.I. & Littlewood, T.D. The role of c-myc in cell growth. *Curr. Opin. Genet. Dev.* **3**, 44-49 (1993).
3. Packham, G. & Cleveland, J. c-Myc and apoptosis. *Biochim. Biophys. Acta* **1242**, 11-28 (1995).
4. Henniksson, M. & Lüscher, B. Proteins of the Myc network: essential regulators of cell growth and differentiation. *Adv. Cancer Res.* **68**, 109-182 (1996).
5. Eilers, M., Picard, D., Yamamoto, K.R. & Bishop, J.M. Chimaeras of myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. *Nature* **340**, 66-8 (1989).
6. Heikkilä, R. *et al.* A c-myc antisense oligodeoxynucleotide inhibits entry into S phase but not progress from G0 to G1. *Nature* **328**, 445-448 (1987).
7. Holt, J.T., Redner, R.L. & Nienhuis, A.W. An oligomer complementary to c-myc mRNA inhibits proliferation of HL-60 promyelocytic cells and induces differentiation. *Mol. Cell. Biol.* **8**, 963-973 (1988).
8. Sklar, M.D. *et al.* Depletion of c-myc with specific antisense sequences reverses the transformed phenotype in ras oncogene-transformed NIH 3T3 cells. *Mol. Cell. Biol.* **11**, 3699-3710 (1991).
9. Sawyers, C.L., Callahan, W. & Witte, O.N. Dominant negative MYC blocks transformation by ABL oncogenes. *Cell* **70**, 901-10 (1992).
10. Hanson, K.D., Shichiri, M., Follansbee, M.R. & Sedivy, J.M. Effects of c-myc expression on cell cycle progression. *Mol. Cell. Biol.* **14**, 5748-5755 (1994).
11. Askew, D.S., Ashmun, R.A., Simmons, B.C. & Cleveland, J.L. Constitutive c-myc expression in an IL-3-dependent myeloid cell line suppresses cell cycle arrest and accelerates apoptosis. *Oncogene* **6**, 1915-22 (1991).
12. Evan, G.I. *et al.* Induction of apoptosis in fibroblasts by c-myc protein. *Cell* **69**, 119-128 (1992).
13. Meichle, A., Philipp, A. & Eilers, M. The functions of Myc proteins. *Biochim. Biophys. Acta* **1114**, 129-46 (1992).
14. Stone, J. *et al.* Definition of regions in human c-myc that are involved in transformation and nuclear localization. *Mol. Cell. Biol.* **7**, 1697-1709 (1987).
15. Kato, G.J., Barrett, J., Villa-Garcia, M. & Dang, C.V. An amino-terminal c-Myc domain required for neoplastic transformation activates transcription. *Mol. Cell. Biol.* **10**, 5914-5920 (1990).
16. Gupta, S., Seth, A. & Davis, R.J. Transactivation of gene expression by Myc is inhibited by mutation at the phosphorylation sites Thr-58 and Ser-62. *Proc. Natl. Acad. Sci. USA* **90**, 3216-3220 (1993).
17. Seth, A., Gupta, S. & Davis, R.J. Cell cycle regulation of the c-Myc transcriptional activation domain. *Mol. Cell. Biol.* **13**, 4125-4136 (1993).
18. Lutterbach, B. & Hann, S.R. Hierarchical phosphorylation at N-terminal transformation-sensitive sites in c-Myc protein is regulated by mitogens and in mitosis. *Mol. Cell. Biol.* **14**, 5510-5522 (1994).
19. Hoang, A.T. *et al.* A link between increase transforming activity of lymphoma-derived MYC mutant alleles, their defective regulation by p107, and altered phosphorylation of the c-Myc transactivation domain. *Mol. Cell. Biol.* **15**, 4031-4042 (1995).
20. Li, L., Nerlov, C., Prendergast, G., MacGregor, D. & Ziff, E.B. c-Myc activates and represses target gene through the E-box Myc binding site and the core promoter region respectively. *EMBO J.* **13**, 4070-4079 (1994).
21. Papas, T.S. & Lautenberger, J.A. Sequence curiosity in v-myc oncogene. *Nature* **318**, 237 (1985).
22. Bhatia, K. *et al.* Point mutations in the c-Myc transactivation domain are common in Burkitt's lymphoma and mouse plasmacytomas. *Nature Genet.* **5**, 56-61 (1993).
23. Yano, T. *et al.* Clustered mutations in the second exon of the MYC gene in sporadic Burkitt's lymphoma. *Oncogene* **8**, 2741-8 (1993).
24. Gu, W., Bhatia, K., Magrath, I.T., Dang, C.V. & DallaFavera, R. Binding and suppression of the myc transcriptional activation domain by p107. *Science* **264**, 251-254 (1994).
25. Beijersbergen, R.L., Hijmans, E.M., Zhu, L. & Bernards, R. Interaction of c-Myc with the pRb-related protein p107 results in inhibition of c-Myc-mediated transactivation. *EMBO J.* **13**, 4080-4086 (1994).
26. Vojtek, A.B., Hollenberg, S.M. & Cooper, J.A. Mammalian ras interacts directly with the serine/threonine kinase Raf. *Cell* **74**, 205-214 (1993).
27. Pulverer, B.J. *et al.* Site-specific modulation of c-Myc cotransformation by residues phosphorylated in vivo. *Oncogene* **9**, 59-70 (1994).
28. Bianchi, M.W., Plyte, S.E., Kreis, M. & Woodgett, J.R. A Saccharomyces cerevisiae protein-serine kinase related to mammalian glycogen synthase kinase-3 and the Drosophila melanogaster gene shaggy product. *Gene* **134**, 51-6 (1993).
29. Pawson, T. & Gish, G.D. SH2 and SH3 domains: from structure to function. *Cell* **71**, 359-362 (1992).
30. Lichte, B., Veh, R.W., Meyer, H.E. & Kilmann, M.W. Amphiphysin, a novel protein associated with synaptic vesicles. *EMBO J.* **11**, 2521-2530 (1992).
31. Folli, F. *et al.* Autoantibodies to a 128-kd synaptic protein in three women with the stiff-man syndrome and breast cancer. *N. Engl. J. Med.* **328**, 546-51 (1993).
32. Brown, R.H. in *Principles of Internal Medicine* (eds Isselbacher, K.J.) 1878-1882 (McGraw-Hill, New York, 1994).
33. Bauer, F., Urdaci, M., Aigle, M. & Crouzet, M. Alteration of a yeast SH3 protein leads to conditional viability with defects in cytoskeletal and budding patterns. *Mol. Cell. Biol.* **13**, 5070-5084 (1993).
34. Land, H., Parada, L.F. & Weinberg, R.A. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature* **304**, 596-602 (1983).
35. Ruley, H.E. Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. *Nature* **304**, 602-606 (1983).
36. Rao, L. *et al.* The adenovirus E1A proteins induce apoptosis, which is inhibited by the E1B 19-kDa and Bcl-2 proteins. *Proc. Natl. Acad. Sci. USA* **89**, 7742-7746 (1992).
37. Leder, A., Pattengale, P.K., Kuo, A., Stewart, T.A. & Leder, P. Consequences of widespread deregulation of the c-myc gene in transgenic mice: multiple neoplasms and normal development. *Cell* **45**, 485-95 (1986).
38. Sinn, E. *et al.* Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. *Cell* **49**, 465-75 (1987).
39. Berns, E.M. *et al.* c-myc amplification is a better prognostic factor than HER2/neu amplification in primary breast cancer. *Cancer Res.* **52**, 1107-13 (1992).
40. Hehir, D.J., McGreal, G., Kirwan, W.O., Kealy, W. & Brady, M.P. c-myc oncogene expression: a marker for females at risk of breast carcinoma. *J. Surg. Oncol.* **54**, 207-9 (1993).
41. Kreipe, H. *et al.* Amplification of c-myc but not of c-erbB-2 is associated with high proliferative capacity in breast cancer. *Cancer Res.* **53**, 1956-61 (1993).
42. Watson, P.H., Safneck, J.R., Le, K., Dubik, D. & Shiu, R.P. Relationship of c-myc amplification to progression of breast cancer from in situ to invasive tumor and lymph node metastasis. *J. Natl. Cancer Inst.* **85**, 902-7 (1993).
43. David, C., McPherson, P.S., Mundigl, O. & de Camilli, P. A role of amphiphysin in synaptic vesicle endocytosis suggested by its binding to dynamin in nerve terminals. *Proc. Natl. Acad. Sci. USA* **93**, 331-335 (1996).
44. Gaubatz, S., Meichle, A. & Eilers, M. An E-box element localized in the first intron mediates regulation of the prothymosin α gene by c-myc. *Mol. Cell. Biol.* **14**, 3853-3862 (1994).
45. Born, T., Frost, J., Schönthal, A., Prendergast, G.C. & Feramisco, J. c-Myc and oncogenic ras induce the cdc2 promoter. *Mol. Cell. Biol.* **14**, 5741-5747 (1994).
46. Bello-Fernandez, C., Packham, G. & Cleveland, J.L. The ornithine decarboxylase gene is a transcriptional target of c-MYC. *Proc. Natl. Acad. Sci. USA* **90**, 7804-7808 (1993).
47. Sakamuro, D. *et al.* c-Myc induces apoptosis in epithelial cells by p53-dependent and p53-independent mechanisms. *Oncogene* **11**, 2411-2418 (1995).
48. Negorev, D. *et al.* The Bin1 gene localizes to human chromosome 2q14 by PCR analysis of somatic cell hybrids and fluorescence in situ hybridization. *Genomics* **33**, 329-331 (1996).
49. Cher, M.L. *et al.* Genetic alterations in untreated metastases and androgen-independent prostate cancer detected by comparative genomic hybridization and allelotyping. *Cancer Res.* **56**, 3091-3102 (1996).
50. Hayata, I. *et al.* Chromosomal aberrations observed in 52 mouse myeloid leukemias. *Cancer Res.* **43**, 367-373 (1983).
51. Jahner, D. & Hunter, T. The ras-related gene *rhoB* is an immediate-early gene inducible by v-Fps, epidermal growth factor, and platelet-derived growth factor in rat fibroblasts. *Mol. Cell. Biol.* **11**, 3682-3690 (1991).
52. Murre, C., McCaw, P.S. & Baltimore, D. A new DNA-binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and Myc proteins. *Cell* **56**, 777-783 (1989).
53. Prendergast, G.C., Lawe, D. & Ziff, E.B. Association of Myn, the murine homolog of Max, with c-Myc stimulates methylation-sensitive DNA binding and Ras cotransformation. *Cell* **65**, 395-407 (1991).
54. Niman, H.L. *et al.* Generation of protein-reactive antibodies by short peptides is an event of high frequency: implications for the structural basis of immune recognition. *Proc. Natl. Acad. Sci. USA* **80**, 4949-4953 (1983).
55. Kelekar, A. & Cole, M. Tumorigenicity of fibroblast lines expressing the adenovirus E1a, cellular p53, or normal c-myc genes. *Mol. Cell. Biol.* **6**, 7-14 (1986).
56. Prendergast, G.C., Hopewell, R., Gorham, B. & Ziff, E.B. Biphasic effect of Max on Myc transformation activity and dependence on N- and C-terminal Max functions. *Genes Dev.* **6**, 2429-2439 (1992).
57. Chen, C. & Okayama, H. High efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7**, 2745-2752 (1987).
58. Harlow, E. & Lane, D. *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1988).
59. Prendergast, G.C. & Ziff, E.B. Mbn1: A novel gelsolin/severin-related protein which binds actin in vitro and exhibits nuclear localization in vivo. *EMBO J.* **10**, 757-766 (1991).
60. Prendergast, G.C. & Cole, M.D. Posttranscriptional regulation of cellular gene expression by the c-myc oncogene. *Mol. Cell. Biol.* **9**, 124-134 (1989).
61. Shiozawa, M. *et al.* Synthesis of human gamma-glutamyl transpeptidase (GGT) during the fetal development of liver. *Gene* **87**, 299-303 (1990).
62. Church, G.M. & Gilbert, W. Genomic sequencing. *Proc. Natl. Acad. Sci. USA* **81**, 1991-1995 (1984).
63. Yamamoto, R., Li, X., Winter, S., Francke, U. & Kilmann, M.W. Primary structure of human amphiphysin, the dominant autoantigen of paraneoplastic Stiff-Man Syndrome, and mapping of its gene (AMPH) to chromosome 7p13-p14. *Human Mol. Genet.* **4**, 265-268 (1995).

**Structural analysis of the human BIN1 gene: evidence for tissue-specific
transcriptional regulation and alternate RNA splicing**

Robert Wechsler-Reya, Daitoku Sakamuro, Jing Zhang, James DuHadaway, and
George C. Prendergast

The Wistar Institute, 3601 Spruce Street, Philadelphia PA 19104

Running title: Human *BIN1* gene structure and regulation

Category: Full length paper (Gene expression)

GenBank Accession Numbers: U68485, U83999-U84004.

Corresponding author responsible for page/proof charges: G.C. Prendergast

Phone: (215) 898-3792

Facsimile: (215) 898-2205

email: prendergast@wista.wistar.upenn.edu

September 3, 1997

Abstract

BIN1 is a putative tumor suppressor that was identified through its interaction with the MYC oncoprotein. To begin to identify elements of BIN1 whose alteration may contribute to malignancy, we cloned and characterized the human BIN1 gene and promoter. Nineteen exons were identified in a region of >54 kb, six of which were alternately spliced in a cell type-specific manner. One alternately spliced exon encodes part of the MYC-binding domain, suggesting that splicing controls the MYC-binding capacity of BIN1 polypeptides. Four other alternately spliced exons encode amphiphysin-related sequences that were included in brain-specific BIN1 species, also termed amphiphysin isoforms or amphiphysin II. The 5' flanking region of BIN1 is GC-rich and lacks a TATA box but directs transcriptional initiation from a single site. A ~0.9 kb fragment from this region was sufficient for basal transcription and transactivation by MyoD, which may account for the high levels of BIN1 observed in skeletal muscle. This study lays the foundation for genetic and epigenetic investigations into the role of BIN1 in normal and neoplastic cell regulation.

Introduction

The identification of tumor suppressor genes in solid tumors is a major goal of cancer research. BIN1 is a novel MYC-interacting protein that has features of a tumor suppressor in certain carcinomas, including those of the breast, liver, cervix, and prostate (1). BIN1 is related to amphiphysin, a neuronal protein that is a paraneoplastic autoimmune antigen associated with breast and lung cancer (2,3), and to RVS167, a negative regulator of the cell cycle in yeast (4). Although widely expressed in normal cells, BIN1 is functionally deleted in ~50% of carcinoma cell lines and primary breast carcinomas examined (1). The human BIN1 gene is located at chromosome 2q14 (5), within a mid-2q region that is deleted in ~42% of metastatic prostate cancers (6). We have hypothesized that BIN1 is a tumor suppressor whose loss contributes to growth deregulation in cancer cells. As a prerequisite to examining this hypothesis, the exon organization, exon-intron boundaries, splice patterns, and promoter of the human BIN1 gene were defined.

Materials and Methods

Cloning and analysis. Genomic clones were isolated from a WI-38 diploid fibroblast λ FIX phage library (a gift of L. Showe) using a BIN1 cDNA probe (1). Six phage inserts designated in Figure 1 were subcloned in pBS+ (Stratagene) and analyzed by extensive restricting mapping and Southern analysis with BIN1 cDNA probes (1). Large scale sequencing of genomic DNA that hybridized to BIN1 cDNA probes, comprising ~20 kb of the ≥ 54 kb locus, was determined using an automated DNA sequencer. The sequence data were assembled manually with assistance from MacVector and AssemblIGN software. Exons and other gene features were identified and/or confirmed by visual inspection or computer-aided comparison of BIN1 cDNAs and ESTs in Genbank, using SIM and ClustalW (pairwise or multiple sequence alignments), TBLASTN (DNA database comparisons), MatInspector, and TESS (promoter binding site identification), accessed through the Baylor College of Medicine Human Genome Center Home Page on the World Wide Web. The Genbank accession number for BIN1 cDNA is U68485 and for BIN1 genomic sequences are U83999 through U84004.

RT-PCR. The substrate for RT-PCR was 2 μ g of total cytoplasmic RNA isolated as described (7). Murine RNAs were a gift of L. Benjamin. Human RNAs were isolated from WI-38 fibroblasts, HeLa cells, or Rh30 rhabdomyosarcoma cells that were cultured as described (1). RNA and 50 pmol oligo-dT (Pharmacia) were added to diethylpyrocarbonate (DEPC)-treated water (final volume 11 μ l), heated 4 min at 70°C, and quenched on ice. RT reactions (20 μ l) was prepared by mixing 4 μ l 5X buffer (250 mM TrisHCl pH 8.3/375 mM KCl/15 mM MgCl₂), 1 μ l 25 mM dNTPs, 2 μ l 0.1 M dithiothreitol (DTT), 1 U RNase inhibitor, and 100 U Moloney murine Leukemia Virus (MoMLV) RT (GibcoBRL). This mixture was incubated 1 hr at 42°C, heated to 94°C for 5 min, and diluted to 100 μ l with DEPC-treated water. Ten microliters of the diluted reaction was used as substrate for 30 cycles of PCR (50 μ l) (45 sec 94°C/45 sec 55°C/1 min 72°C) with 0.5U Taq polymerase (Pharmacia) in 1X buffer/0.2 mM dNTPs/1.5 mM MgCl₂.

Separate PCR reactions were performed to generate 5' (N-terminus; exons 3-7), midsection (exons 6-11), and 3' (C-terminus; exons 11-16) segments of the BIN1 coding region. For amplifying products from human BIN1 message (1), the 5' and 3' primer pairs used were as follows. For the 5' (N-terminus) product, AAGGATCTCCGGACCTACCT (cT7/ext) and CACATTCATCTCCTCAAACACC (ptx7 α); for the midsection product, TGAAGCCAAAATTGCCAAGGC (dT3/ext) and TGGCTGAGATGGGGACTTG (5'ATG99); and for the 3' (C-terminus) product, GGAGAATTCGCGATGCCTGCAAAAGGGAACAAGAGC (99Fsp) and GGACTCGAGTCATGGGACCCTCTCAGTGAAGTTC (99SH3anti). For amplifying products from murine BIN1 message (8), the 5' and 3' primer pairs used were as follows. mNTsen1 (5'-CAGTGCGTCCAGAATTTC) and mNTanti1 (5'-AACACCTTCTGGGCTTTG); mMIDsen1 (5'-AAGCCCAGAAGGTGTTTCGAG) and m5'ATG99 (5'-TGGCTGAGATGGGGACTT); and mCTsen1 (5'-CTGAGATCAGAGTGAACCATG) and mCTanti1 (5'-CACCCGCTCTGTAAAATTTC). Products were fractionated on agarose gels, blotted, and hybridized, or isolated and subcloned for DNA sequencing.

Primer extension. Three pmol of the oligonucleotide primer ACAGCGGAGCCAACTGAC (PEprimer#2) end-labeled with $\gamma^{32}\text{P}$ -ATP was annealed to 10 μg of WI-38 total cytoplasmic RNA for 12 hr at 58°C in hybridization buffer (40 mM PIPES pH 6.4, 1 mM EDTA, 0.4 M NaCl, 80% formamide). The annealed RNA was ethanol precipitated and resuspended in 20 μl RT reaction buffer (see above) plus 50 $\mu\text{g}/\text{ml}$ actinomycin D (BMB). The reaction mixture was incubated 1 hr at 42°C and stopped by the addition of sequencing gel loading buffer. A standard $\alpha^{35}\text{S}$ -dATP DNA sequencing reaction was performed with the same primer using clone p31.2 as the template (see Figure 1). The primer extension and sequencing products were cofractionated on a 12% DNA sequencing gel and autoradiographed for 15 min (extension reaction) or overnight (sequencing reaction).

Transcription assays. pGL-Bgl was generated by subcloning a 886 Bgl II fragment including proximal 5' flanking sequences from p31.2 into the luciferase reporter plasmid pGL2-Basic (Promega). The hosts for transfection, C2C12 cells or 10T_{1/2} cells (a gift of D. Goldhamer), were cultured in DMEM containing 15% fetal calf serum and penicillin/streptomycin. Cells (4×10^4 per 60 mm dish) were transfected (9) with 5 μ g pGL-Bgl or pGL2-basic, 4.5 μ g pSK+ (Invitrogen), and 0.5 μ g CMV- β gal. For MyoD activation, a murine MyoD expression construct under control of the β actin promoter (a gift of D. Goldhamer) was substituted for pSK+. Cells were harvested and processed for luciferase activity two days post-transfection using a commercial kit (Promega).

Results

BIN1 gene structure and exon-intron organization. A physical map of the human BIN1 gene was constructed from a set of phage clones isolated from a WI-38 diploid fibroblast genomic library (see Figure 1). To identify exons and exon-intron boundaries, the DNA sequence determined from six genomic segments (GenBank accession numbers U83999 through U84004) was compared to BIN1 cDNA sequences from several sources, including the original BIN1 clone, RT-PCR products from human RNAs, and the DNA database (1,8,10-12). With the exception of exon 1, all exons were located within a ~38 kb contig. An additional noncontiguous clone contained exon 1 and 5' flanking sequences, with the latter extending ~3 kb upstream of the RNA cap site (see below). Given the structure of this clone, the size of intron 1 would be inferred to be at least 17 kb. Thus, we concluded that the human BIN1 gene spanned a minimum of 54 kb.

The DNA sequence of each exon and proximal introns are shown in Figure 2. Based on the characteristics they encode, the BIN1 exons can be grouped into four sets, termed the BAR (Bin1/Amphiphysin/Rvs167-related), unique, brain-specific, and protein-protein interaction sets, respectively. Exons 1-8 encode the BAR domain of BIN1 (1). In this group, exon 1 included a different 5' UTR and N-terminal coding sequence (MAEMGSKG) compared to the original BIN1 cDNA (MLWNV) (1). The genomic sequence was judged to accurately represent the 5' end of the BIN1 mRNA, because (i) expressed sequence tag (EST) and cDNA sequences identical to the genomic but not the 5' BIN1 cDNA sequence were present in the DNA database; (ii) cDNAs whose structure matched the original cDNA clone could not be identified by RT-PCR in any tissue; and (iii) the 5' end of the cDNA was found to contain an inversion of 64 bp derived from the middle of the cDNA (previously missed because the inversion fortuitously contained a translation initiation site). Exons 9-11 encode a unique region of BIN1 that is functionally undefined and unrelated to amphiphysin and RVS167. The unique-1 (U1) and unique (U2) regions are encoded by exons 9 and 11, respectively, separated by a nuclear localization-like motif encoded by exon 10.

Exons 12A-12D encode amphiphysin-related sequences that were not found in the original BIN1 cDNA (see Figure 2B). These exons are spliced into larger isoforms of BIN1 message detected in brain and muscle, alternately termed amphiphysin isoforms or amphiphysin II (1,10-12) (see below). Exons 13-16 encode the C-terminal region of BIN1 implicated in protein-protein interactions. Exons 13-14 and 15-16 encode the Myc-binding domain (MBD) and the Src homology 3 (SH3) domain, respectively, the latter of which is also a feature of amphiphysin and RVS167 (1).

Alternate splicing of *BIN1* RNA. To examine patterns of BIN1 splicing, RT-PCR was performed using RNAs isolated from three human cell lines, WI-38 diploid fibroblasts, HeLa cervical carcinoma, and Rh30 rhabdomyosarcoma (a muscle tumor line). The observations in human cells were extended using RNAs isolated from a set of normal murine tissues. All cells and tissues examined were previously shown to express BIN1 RNA by Northern analysis (1). Oligonucleotide primers derived from BIN1 sequences were used for RT-PCR of fragments spanning exons 3-7 (5' end), 7-11 (midsection), and 11-16 (3' end). For analysis of murine RNAs, oligonucleotide primer sequences were derived from the sequence of SH3P9, a murine BIN1 cDNA (GenBank accession U60884; ref. 8). Products from these reactions were fractionated on agarose gels, blotted, and hybridized to a BIN1 cDNA probe, or subcloned and sequenced.

- Amplification of the 5' end of BIN1 yielded a single product in all cell lines examined, indicating this region was not subjected to alternate splicing in these cells. In contrast, amplification of the central and 3' regions revealed several alternate splicing events. In the central region, two products of similar abundance were observed in WI-38 fibroblasts that differed in the presence or absence of exon 10 sequences. Messages including exon 10 sequences were not detected in BIN1 messages from any of the other tissues or cell lines examined, suggesting this splice form was relatively uncommon and thus regulated. Amplification of the 3' end revealed

additional splice forms. Two products of similar abundance were detected in all cell types which differed in the presence of exon 13 sequences, which encodes part of the Myc-binding domain (MBD) (1,13). The coordinate appearance of each species suggested that exon 13 was alternately spliced but in a unregulated fashion. Additional species that included sequences derived from exons 12A-12D were detected in murine brain (E9.5 RNA also included one of these species). Interestingly, while exon 12A-12D sequences were not detected in any other normal murine tissues, exon 12A was included in RNA species in each of the established human cell lines. It was unclear whether the difference in exon 12A splicing reflected tissue-specific regulation, cell line establishment, or neoplastic transformation. Nevertheless, taken together with the brain-specific events, this observation suggested that splicing of exons 12A-12D was uncommon in most tissues and thus may be regulated.

To determine which combinations of exons appeared in various BIN1 RNAs, we performed RT-PCR using exon 9 and 16 primers (which span all the alternately spliced exons) and subcloned and sequenced the products. We confined this analysis to RNAs isolated from WI-38 and HeLa cells, where exons 10, 12A, and 13 are alternately spliced, to focus on the events in proliferating rather than postmitotic cells. The results, which are summarized in Figure 3B, showed that seven of the eight RNA species theoretically possible in these cell lines were in fact generated (the one that was not detected was the -10+12A-13 species). We concluded that exons 10, 12A-12D, and 13 of BIN1 were alternately spliced and that splicing of exons 10 and 12A-12D splicing was likely to be regulated.

Definition of 5' flanking sequences sufficient for basal transcription and MyoD activation. Definition of the BIN1 promoter was of interest for two reasons. First, previous work suggested that epigenetic mechanisms might underlie the loss of BIN1 expression in breast tumor cells (1). Therefore, characterization of the BIN1 promoter would permit an examination of tumor DNA for alterations in DNA methylation or transcription factor interactions

which might account for loss of expression. Second, we have observed previously that BIN1 is expressed at high levels in skeletal muscle and murine C2C12 myoblasts (1,14). For this reason, we predicted that the BIN1 promoter might be activated by MyoD, a master regulator of muscle cell differentiation (15).

In order to identify the BIN1 promoter, it was first necessary to pinpoint the site(s) of transcription initiation. To this end, primer extension analysis was performed on RNA from WI-38 diploid fibroblasts. By comparing the genomic sequence to that of a murine BIN1 cDNA (8), which has a long 5' UTR, a primer that was likely to hybridize within 100 nt of the RNA cap site was chosen. RT-mediated primer extension yielded a 33 nt product (see Figure 4). Together with the DNA sequence of the 5' flanking region generated by this primer, we were able to map the 5' end of BIN1 RNA in WI-38 cells to the guanine residue designated +1 in Figure 5.

Determination of the genomic sequence upstream of the RNA cap site indicated that the 5' flanking region was GC-rich and lacked a TATA box but contained a consensus binding site for TATA-binding protein (TBP) at -79 (see Figure 5). Supporting the possibility that MyoD may regulate BIN1, a consensus recognition site for MyoD was located at -238. Consistent with a possible promoter function, computer search algorithms identified consensus sites for several other transcription factors in this region (data not shown). Finally, among the 694 nucleotides upstream of exon 1, ~18% were composed of CpG dinucleotides, indicating the presence of a CpG island typical of many TATA-less promoters. These observations suggested that the 5' end of the BIN1 gene we cloned might contain a functional promoter.

The transcriptional potential of the 5' flanking region was tested in a transient transfection assay. An 886 bp Bgl II restriction fragment was cloned into the luciferase reporter plasmid pGL2-Basic, allowing transcription to be initiated at the BIN1 cap site. The resulting plasmid, pGL2-Bgl, was transfected into C2C12 myoblasts, which express high levels of endogenous Bin1

RNA (14). As shown in Figure 5C, within two days after transfection, pGL2-Bgl exhibited ~100-fold greater activity than the control plasmid pGL2-Basic (see Figure 6A).

To determine whether pGL2-Bgl included sequences that were sufficient for regulated expression, the plasmid was introduced with or without a MyoD expression vector into 10T_{1/2} fibroblasts (which do not express MyoD but in response to it differentiate into myoblasts). As a positive control for MyoD responsiveness, a second set of transfections used a luciferase reporter driven by a mutated ornithine carboxylase promoter (ODCΔSmut-luc) containing a MyoD E box response element (J. Cleveland, unpublished results). We observed that the activity of both reporters was increased up to ~7-fold the basal level by MyoD cotransfection (see Figure 6B). The effect was dose-dependent because higher ratios of MyoD:reporter plasmids increased reporter activity. We concluded that the 5' flanking sequences of the BIN1 gene constituted a promoter sufficient for directing transcription in myoblasts.

Discussion

We have characterized the structure and some of the regulatory features of the human BIN1 gene. Nineteen exons were identified within a ≥ 54 kb region of DNA previously mapped to chromosome 2q14 (5). The primary BIN1 transcript was found to be extensively spliced, resulting in at least seven different species in proliferating cells and an even larger number in postmitotic cells of the brain. Characterization of the BIN1 promoter defined a region sufficient to direct inducible transcription in muscle cells, where BIN1 is highly expressed. Thus, BIN1 is subjected to tissue-specific regulation at the levels of transcription and splicing.

Exons 10 and 13 were two of the three exons found to be alternately spliced in proliferating cells. Exon 10 splicing was relatively uncommon, since it appeared only in messages from WI-38 in addition to skeletal muscle (the source of the original BIN1 cDNA). Exon 10 encodes a basic amino acid-rich region that closely resembles a nuclear localization signal (NLS). However, this region may not act as an NLS because we have observed recently that its presence is neither necessary nor sufficient for nuclear localization (14,16). Therefore, exon 10 splicing probably has other implications. Exon 13 splicing was ubiquitous but apparently unregulated, since an approximately similar quantity of +13 and -13 RNA species were detected in all cells examined. Since this exon encodes a significant part of the MBD, it is likely that its alternate splicing affects the MYC-interacting potential of BIN1. Taken together, an interesting implication of our results is that there are two classes of BIN1 polypeptides that exist in cells, one that can interact with MYC and one that can not.

Four exons identified in this study, 12A-12D, were not included in the original BIN1 cDNA but were detected by RT-PCR in a subset of messages in brain. The existence of brain-specific exons was suggested previously by Northern analysis, which revealed a larger message(s) in brain in addition to the ubiquitously expressed smaller species (1). Our findings confirmed

those of others who have recently identified exon 12A-12D sequences in brain and muscle cDNA species, alternately termed amphiphysin isoforms or amphiphysin-II (10-12). Another cDNA species identified by these workers imply the presence of an additional 93 bp brain-specific exon in intron 6 (which is unrelated to amphiphysin or RVS167); however for unknown reasons we were unable to confirm its presence in the expected location either by RT-PCR or direct DNA sequencing. Exons 12A-12D encode sequences related to amphiphysin, so their introduction would be expected to increase the amphiphysin-like character of BIN1. Alternate splicing of exon 12A-12D in a subset of brain messages may therefore provide a mechanism to augment or vary certain amphiphysin functions in neurons, while retaining BIN1 functions in the same cell.

Interestingly, we found that exon 12A was spliced into a subset of messages in the human cell lines WI-38, HeLa, and Rh-30, but not into messages in normal nonneuronal tissues. The significance of exon 12A splicing in these cells is unclear. However, since we did not detect the +12A-13 isoform in cells, an interesting possibility is that +12A and -13 isoforms are functionally redundant (that is, they each lack the ability to interact with and inhibit the oncogenic properties of MYC). If so, the appearance of +12 isoforms in WI-38 and HeLa cells may reflect an aberrant splicing event that relieves MYC down regulation by BIN1 (1), thereby promoting immortalization or establishment. In general, the extensive splicing we have documented in BIN1 opens the possibility that splice site mutations or altered splicing via epigenetic mechanisms may be germane to tumorigenesis. Two important goals of future work will be to (i.) assess the activities of different splice forms of BIN1 for MYC interaction, cell localization, and inhibition of neoplastic cell growth, and (ii.) determine whether there are altered splice patterns in tumor cells that could compromise the growth inhibitory activity of BIN1.

The BIN1 promoter is characterized by a high CpG content but otherwise exhibits the features of a housekeeping promoter. We showed that the muscle determination factor MyoD can upregulate the BIN1 promoter and identified an E box site that might mediate MyoD-induced

activation. Transcriptional activation by MyoD and/or other helix-loop-helix proteins may contribute to the strong upregulation of BIN1 levels in differentiated neurons and muscle cells (1,10). Since the BIN1 promoter is rich in CpG residues, it is highly susceptible to the alterations in CpG methylation status which are common in cancer cells and which form the basis for loss of some tumor suppressors such as p16INK4 in lung cancers (17). Other mechanisms by which promoter activity might be altered in cancer cells include genetic mutations or epigenetic changes in the activity of transcriptional regulatory factors. The stage is now set to determine the basis for the frequent loss of BIN1 expression in certain solid tumors such as breast carcinoma (1).

Acknowledgments

We thank R. Buccafusca and X. Gong for technical assistance. For providing reagents, we are grateful to L. Benjamin for murine RNAs, J. Cleveland for the MyoD-responsive ODC Δ mutS-luc reporter plasmid, D. Goldhamer for murine C2C12 and 10T $_{1/2}$ cells and a β actin promoter-driven MyoD expression vector, and L. Showe for the WI-38 λ FIX genomic library. We acknowledge the Wistar Institute Core Facilities for oligonucleotide synthesis and DNA sequencing. This work was supported by grants DAMD17-96-1-6324 from the US Army Breast Cancer Research Program and CN160 from the American Cancer Society (G.C.P.). R.W.-R. is an MRC Canada Postdoctoral Fellow. D.S. is the recipient of the Christopher Davis Memorial Fellowship. G.C.P. is the recipient of an American Cancer Society Junior Faculty Award and is a Pew Scholar in the Biomedical Sciences.

References

1. Sakamuro, D., Elliott, K., Wechsler-Reya, R., and Prendergast, G. C. (1996) *Nature Genet.* **14**, 69-77
2. David, C., Solimena, M., and De Camilli, P. (1994) *FEBS Lett.* **351**, 73-79
3. Dropcho, E. J. (1996) *Ann. Neurol.* **39**, 659-667
4. Bauer, F., Ordaci, M., Aigle, M., and Crouzet, M. (1993) *Mol. Cell. Biol.* **13**, 5070-5084
5. Negorev, D., Reithman, H., Wechsler-Reya, R., Sakamuro, D., Prendergast, G. C., and Simon, D. (1996) *Genomics* **33**, 329-331
6. Cher, M. L., Bova, G. S., Moore, D. H., Small, E. J., Carroll, P. R., Pin, S. S., Epstein, J. I., Isaacs, W. B., and Jensen, R. H. (1996) *Canc. Res.* **56**, 3091-3102
7. Prendergast, G. C., and Cole, M. D. (1989) *Mol. Cell. Biol.* **9**, 124-134
8. Sparks, A. B., Hoffman, N. G., McConnell, S. J., Fowlkes, D. M., and Kay, B. K. (1996) *Nat. Biotech.* **14**, 741-744
9. Chen, C., and Okayama, H. (1987) *Mol. Cell. Biol.* **7**, 2745-2752
10. Butler, M. H., David, C., Ochoa, G.-C., Freyberg, Z., Daniell, L., Grabs, D., Cremona, O., and De Camilli, P. (1997) *J. Cell Biol.* **137**, 1355-1367
11. Ramjaun, A. R., Micheva, K. D., Bouchelet, I., and McPherson, P. S. (1997) *J. Biol. Chem.* **272**, 16700-16706
12. Tsutsui, K., Maeda, Y., Tsutsui, K., Seki, S., and Tokunaga, A. (1997) *Biochem. Biophys. Res. Comm.* **236**, 178-183
13. Elliott, K., Sakamuro, D., Du, W., and Prendergast, G. C. (1997) *Oncogene*, under revision.
14. Wechsler-Reya, R., Elliott, K., and Prendergast, G. C. (1997) *Mol. Cell. Biol.*, under revision.

15. Tapscott, S. J., Davis, R. L., Thayer, M. J., Cheng, P.-F., Weintraub, H., and Lassar, A. B. (1988) *Science* **242**, 405-411
16. Wechsler-Reya, R., Elliott, K., Herlyn, M., and Prendergast, G. C. (1997) *Canc. Res.* **57**, 3258-3263
17. Merlo, A., Herman, J. G., Mao, L., Lee, D. J., Gabrielson, E., Burger, P. C., Baylin, S. B., and Sidransky, D. (1995) *Nat. Med.* **1**, 686-692
18. Yamamoto, R., Li, X., Winter, S., Francke, U., and Kilimann, M. W. (1995) *Human Mol. Genet.* **4**, 265-268
19. Quandt, K., Frech, K., Haras, H., Wingender, E., and Werner, T. (1995) *Nuc. Acids Res.* **23**, 4878-4884

Figure Legends

Figure 1. Physical map of human BIN1 gene. Exons are numbered and shaded to indicate their relative coding position within the BIN1 polypeptide. BAR, U1, NLS, U2, MBD, and SH3 represent the Bin/Amphiphysin/RVS167 homology region, unique-1 region, nuclear localization motif, unique-2 region, MYC-binding domain, and Src homology-3 domain, respectively (1,16). U1 and U2 are functionally undefined sequences which are unique to BIN1 and not conserved in amphiphysin or RVS167. Exons 12A-12D were absent from the original BIN1 cDNA but were detected in brain-specific messages by RT-PCR, sequence analysis, and database comparisons (see text). The triangle above the BIN1 polypeptide diagram indicates the position where residues encoded exons 12A-12D are inserted. The dark hatched bar above exon 1 depicts the 886 bp Bgl II promoter fragment analyzed in this study. Dashed lines represent the inserts of various phage clones.

Figure 2. Exon-intron structure. (A.) *Exon and proximal intron sequences.* The figure is read left to right, with complete exons shown in the left panel and introns following each shown in the right panel. Register is 50 bp per line. Sizes of intron gaps noted are exact, if sequenced; otherwise estimated from restriction mapping. (B.) *Similarity between exon 12A-12D and amphiphysin sequences.* Alignment of the predicted open reading frames of BIN1 exons with aa 298-239 of human amphiphysin (18).

Figure 3. Alternate splicing of exons 10, 12A-12D, and 13. (A.) *RT-PCR.* Human cell lines (top panel) and normal murine tissues (bottom panel) were analyzed. Total cytoplasmic RNA was isolated and used as template for RT-PCR as described in the Materials and Methods. Products were fractionated on 1.5% agarose gels, stained with ethidium bromide, and photographed. The exons present in the PCR bands was confirmed by DNA sequencing. (B.) *Summary of BIN1 splice products.* Splice forms identified in WI-38 and HeLa cells are depicted.

The relative location of the PCR products within the BIN1 cDNA are illustrated at the bottom of the figure; the 5' product includes exons 3-7; the midsection product, exons 7-11; and the 3' product, exons 11-16. Exons 10 and 13 but not 12A were included in the original BIN1 cDNA isolated from muscle cells (1).

Figure 4. RNA cap site mapping. Primer extension using WI-38 RNA was performed as described in the Materials and Methods. A DNA sequencing reaction was performed in parallel using the same primer and genomic clone p31.2 as template. The two panels are from different exposures of the same autoradiograph.

Figure 5. Structure of the 5' flanking region. The DNA sequence of the 886 bp Bgl II-Bgl II promoter fragment noted in Figure 1 is presented. The RNA cap site is indicated by +1; exon 1 is shown in upper case letters. Single underlining indicates the antisense sequence of the oligonucleotide used for the primer extension experiment. Double underlining indicates a potential binding site for TBP at -79, identified by MatInspector (19), and a single E box consensus binding site for MyoD at -237, identified by visual inspection.

Figure 6. Promoter activity of 5' flanking sequences. (A.) *Basal transcription.* C2C12 cells were transfected with 5 µg of the luciferase reporters indicated and processed 48 hr later as described in the Materials and Methods. pGL2-Basic is the no insert control vector. pGL-Bgl contains the ~0.9 kb Bgl II-Bgl II fragment whose sequence is shown in Figure 5. (B.) *MyoD activation.* 10T_{1/2} cells were transfected and processed as above except that the transfected DNAs included the indicated amounts of a MyoD expression vector. ODCΔmut-luc is a positive control reporter for MyoD responsiveness (see text). The data represent the average of two trials.

Fig.1

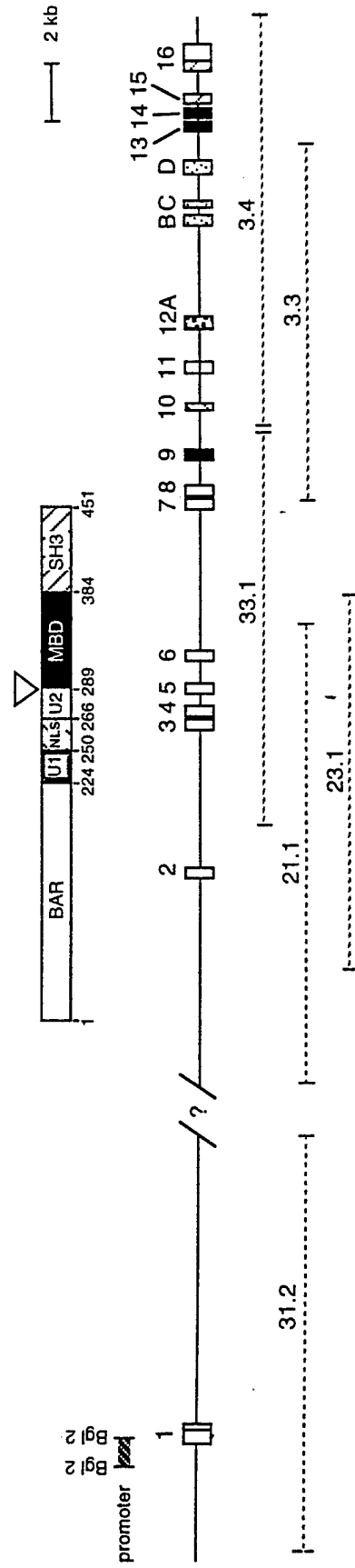


Fig. 2A)

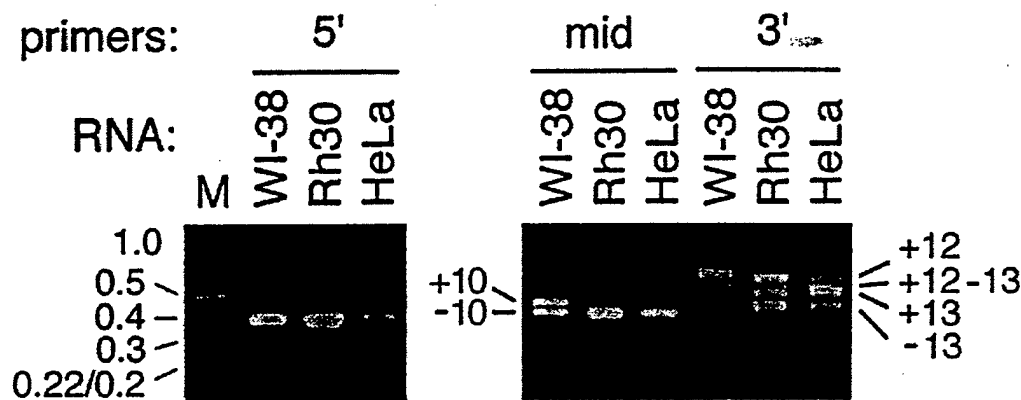
No.	EXON	INTRON
1	GCACGCTCCGCGCTCCGTCAGTTGGCTCCGCTGTCCGGTTCGCGCGCGTGG AGCGGCAAGCCGGTCTTGACAGCGCGCGCGCGCTTGGGGCTGGGAGCGCG CGCGCAAGATCTCTCCCGCGCGAGAGCGGCCCTTGCACACGGGCGAGGC CTCGCGCCCGATGGCAGAGATGGCGAGTAAAGGGGTGACGCGCGGAAAGA TCGCGCAACCACTGACAGAGAAGCTCACCCGCGCGCAGGAGAAG	gtgagcgagccggagcccgacgagccgcggaggtcccagcgccgcgaggc ...intron 1 (217 kb)... cactntctcttccaaggtaacactctccccctctctntnggnttcggcag
2	GTCTCTCAGCAGCTGGGGAAGGACAGATGAGACCAAGGATGAGCAGTTGA GCAGTGCCTCCAGAATTCAACAAGCAG	attgggtgagctgtgggtgccccttgcccttagggagtttgtgaaaattcgg ...intron 2 (-5 kb)... ggactgggtgtgccaccagtccttgacctggtcctttgctctctcttttgc gtgaggaggcaggcagaagcgacctggcccgctgtagagctgggtgggtgc ...intron 3 (16 bp)... ggacaagcgctatcagtgaggagcccaatacttcttctgccccacag
3	CTGACGGAGGGCACCCGGCTGCAGAAGATCTCCGGACCTACCTGGCCCTC CGTCAAG	gtgagcatgggacaggtggggcctgccccttctcagaggggcccctctgggtctc ...intron 4 (362 bp)... ggtgtggccagccctcctggtggcactgctgctgctcttgccctgtctcag gtgagagaccacttgacatctgcctgtctgctgctggagggaactgg ...intron 5 (769 bp)... ctcagcaggggtgcccagggccccgcttagtgccctcctctgtctgccccag
4	CCATGCACGAGGCTTCCAAGAAGCTGAATGAGTGTCTGCGAGAGGTGTAT GAGCCCGATTGGCGCGGCGAGGATGAGGCAACAAGATCGCAGAG	gtgagggctggagcgggtggggcgagcgtctgagggcgctgagagggctgagaggg ...intron 6 (-3.5 kb)... gggctggctgggacgcccacagaggggaatgaccctgctgccccccag gtgagggccggcagcgtgcccagcctgctggtggggcagtgctcagctctgcg ...intron 7 (186 bp)... tgtcctgacctgctctgttacctgccccccatccttccgccccttctgtag
5	AACAACGACCTGCTGTGGATGGATTACCAACAGAAGCTGGTGGACAGGC GCTGTGCACCATGAGACAGTACCTGGGCCAGTTCGCCGACATCAAG	gtagggccatggggagcccctctgaggggccacacccaccctggccgaggg ...intron 8 (1275 bp)... ccgtttgtggccttggaggccccccacctctcactgtctctctctgcag gtgcgtgccccggagagccctggcccccctgactgtgtgcagggcagggggc ...intron 9 (1373 bp)... cctggccttcttcagaaatgaagcctccacctccgctccgtccccacag
6	TCACGCATTGCCAAGCGGGGGCGCAAGCTGGTGGATACGACAGTGCCCGG CACCATCTACGAGTCCCTTCAAACTGCCAAAAGAGAGATGAAGCCAAAT TGCCAAAG	gtaccggcagtgagtgctgctggggggggcgagagggccgcccgtccgtgct ...intron 10 (1339 bp)... tgtgctctggcccccaaacactgggctcccccttcttctgctccccacag gtagggagngaaacttttggtagaagggccccaagcctccatcctantcgt ...intron 11 (1408 bp)... gctctgtgctgcttcccgccgccccctcaccgcgcccgcagccacagc
12A	CTCCGGAAGAGGCCACACAGTCCCTCGGCTCCCAAACACACCCCGTCCAA GGAAGTCAAGCAGGAGAGATCTCTACGCTGTGTTGAGGACACGTTGTTC CTGAGATCTGAGCTGACCAACCCCTCCCAAG	gtcagccgcccgcgcgcgcccagcgtctctctctctctgctgctcag ...intron 12A (-4 Kb)... ccagcttaaacactgactgtctctctctgctgctgctgctgctgccaag gttggtgtgcccaccactgcccattggggccaccagcttccaggtgccc ...intron 12B (460 bp)... tctctaaectctgtgctaactgtccttctgcccctcactgtgcgctcag
12B	TTTGAAGGCCCCGGGGCTTATCTCGGAGCAGGCGAGTCTGCTGGAACCTGA CTTTGACCCCTTCCGCGCGGTGACGAGCCCTGTGAAGCACCACGCCCC CTGCTCAG	gttaagctgcaactgtctcttctgaccaccctgggggaaccactcttctc ...intron 12C (1062 bp)... ccccggcctccccggccccctcctgttctctctctgctggcctgttaccag gtaagtgcccaactccagcccctgtctggttgtccccagtgctctagggg ...intron 12D (1012 bp)... tggtgtgtctctgtctctgtgtgtgaccccaagccggcatttatgttgag
12C	TCAATTCCATGGGACCTCTGGGAG	gtaagacagcagggacaagcctggtcttctctctcctcctcctgcccgcgt ...intron 12 (142 bp)... tgtcctggaccacttctcctgctcagctgaccccgtaactgtgccaccag gtgagcccaagcgtctgactgtgcagtcctcctcggtgccctgtgggga ...intron 13 (179 bp)... gggctggaggtgggaggaaggtctgacttgcatccgcatcctctgcag
12D	CCCACAGAGATCCAGCGCGCAGCTGCTTCCGGGAGGCCAGCGCTGC CGAGGGCACCTTTGCTGTGTCTGGCCAGCCAGACGCGCCGAGCGGGGC CTGCCCAA	gtgagggctgggtggggccccacacncanggggaccaccnngcatcct ...intron 14 (1687 bp)... gggtgganccctgtctcctcctgtgcgacctgntgctctgcccctcag aaaaacaaatgaaacaaaaaaaatgataaaaactctcagaaaaactgtg gtgtatttgtctctctctctctgttcgtgagtgcggtggaacggtgtn atctgtgcttctctactagatgttctgcccccgagcccgctgacct nccgctggtgcaacacagggctgacccccctgccccctgggtgtcttagg gggtgtcactgtggangtcangagctgggggctgggttaaggggctgtgc caccactctctgtccanaaatcttgctnactgccccctaaact ...3'
13	CCAGCAGAGGGCTCGGAGGTGGCGGGTGGGACCAACCTGCGGCTGGAGC CCAGGAGCCAGGGAGACGCGCGCAAGTGAAGCAGCCTCC	
14	AGCTCTCTTCTGCTGTCTGCTGGTGGAGACCTTCCAGCAACTGTGAATGG CACCGTGGAGGGCGCGCTGTGGGGCGGGCGCTTGGACCTGCCCCAGGTT TCATGTTCAAG	
15	GTACAGGCCACGACGACTACAGGGCACTGACACAGACGAGCTGCAGCT CAAGGCTGGTGATGTGGTGCTGTTGATCCCTTCCACAACCTGAAGAGC AG	
16	GATGAAGGCTGGCTCATGGGCGTGAAGGAGCGACTGGAACACGACAA AGAAGCTGGAGAAGTGCCTGGCGCTTCTCCCGAGAACTTCACTGAGAGGG TCCCATGACGGCGGGGCGCAGGACGCTTCCGGCGCTGTGAAGAACACTTC CTCCGGAATAATGTGTGCTTTTCTTTTGTGTTTCTGTTTTCATCT TTTTGAAGAGCAAAGGGAATCAAGAGAGAGACCCCGCAGGAGGGGCGT TCTCCCAAAGTTTAGTGCTTTTCCAAAGAGCGCGCTCCCGGCAAGTCCG CGGAATTCAACGATGCTCTGAAAGCTCTGTGTCTCTAGTGTAGTGT TGGCGCCCTTGCCTGTGCCCGATGTGTGAGCTGGCCGCGAGGGCGGGCT GGGCTGCCCGAGCTCACTAATCTAAAGCTTCCGCGCGCACCCCGG GGAAGGCTCTCTTTTCTGCGAGCTGCTGTGGGTGGGGCCGACACCA GCCTAGCCCTGCTGCCGCCCGCAGACGGCTGTGTGCTGTTTGAATAAA TCTTATGTTTC	

Fig. 2B)

amphiphysin	297	TRKGPPVPPLPKVTPTKELQQENIISFFEDNFVPEISVTTPSQ	339
		: : : : :	
exon 12A ORF		LRKGPPVPP-PPHTPSKEVEQKQILSLFEDTFVPEISVTTPSQ	
amphiphysin	340	NEVPEV-KKEETLLDLDFDPFKPEVTPAGSAGVTHSPHSQ	378
		: : : : : :	
exon 12B ORF		FEAPGPFSEQASLLDLDFDPLPPVTSPVKAP----TPSGQ	
amphiphysin	379	TLPWDLWT	386
		:	
exon 12C ORF		SEPWDLWE	
amphiphysin	422	CNLAESEQAPPTPEPKAEPLAAVTPAVGLDLGHDTR	457
		: : : :	
exon 12D ORF		PTESPAGSLPSGEPSSAAEGTFAVSHPSQTAEPPGAQ	

Human cell lines

Fig.3A



Murine tissues

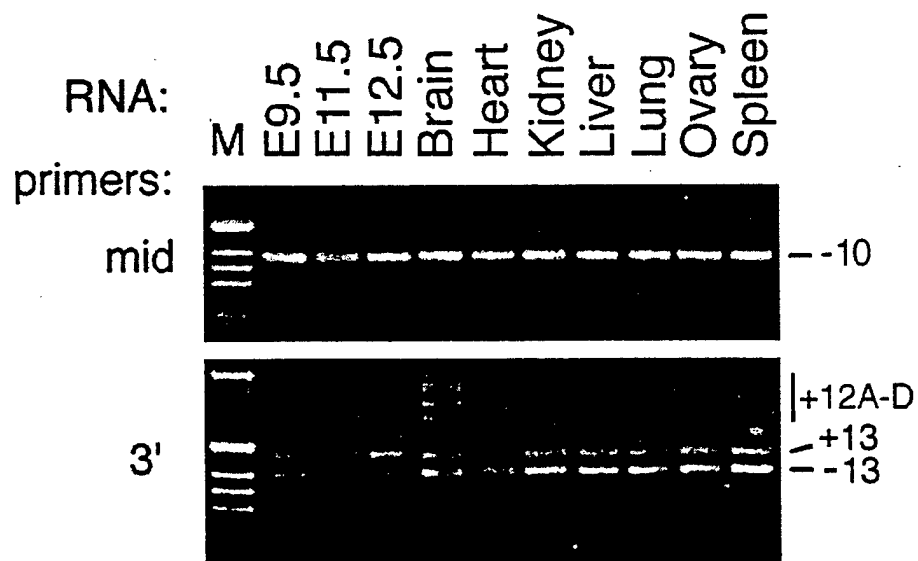


Fig. 3B

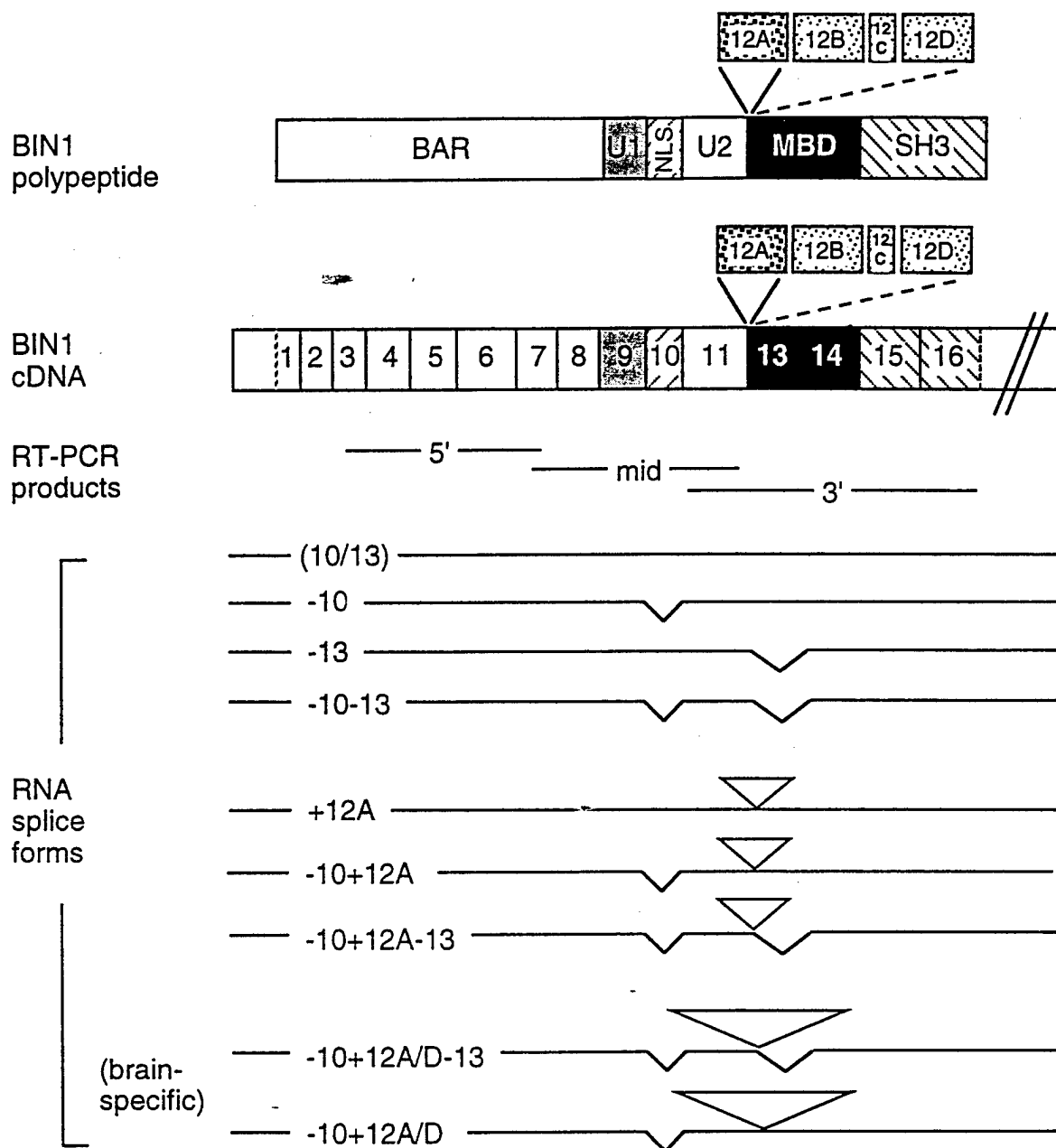


Fig.4

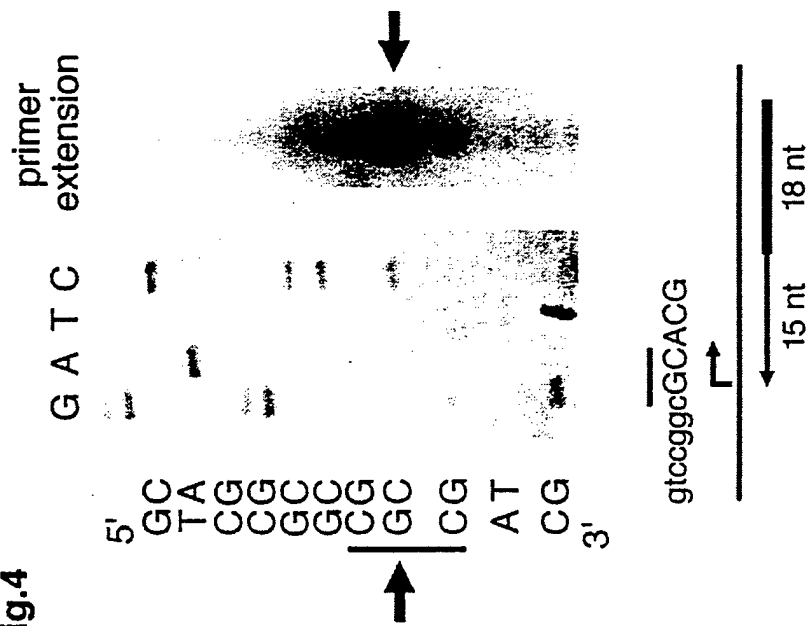


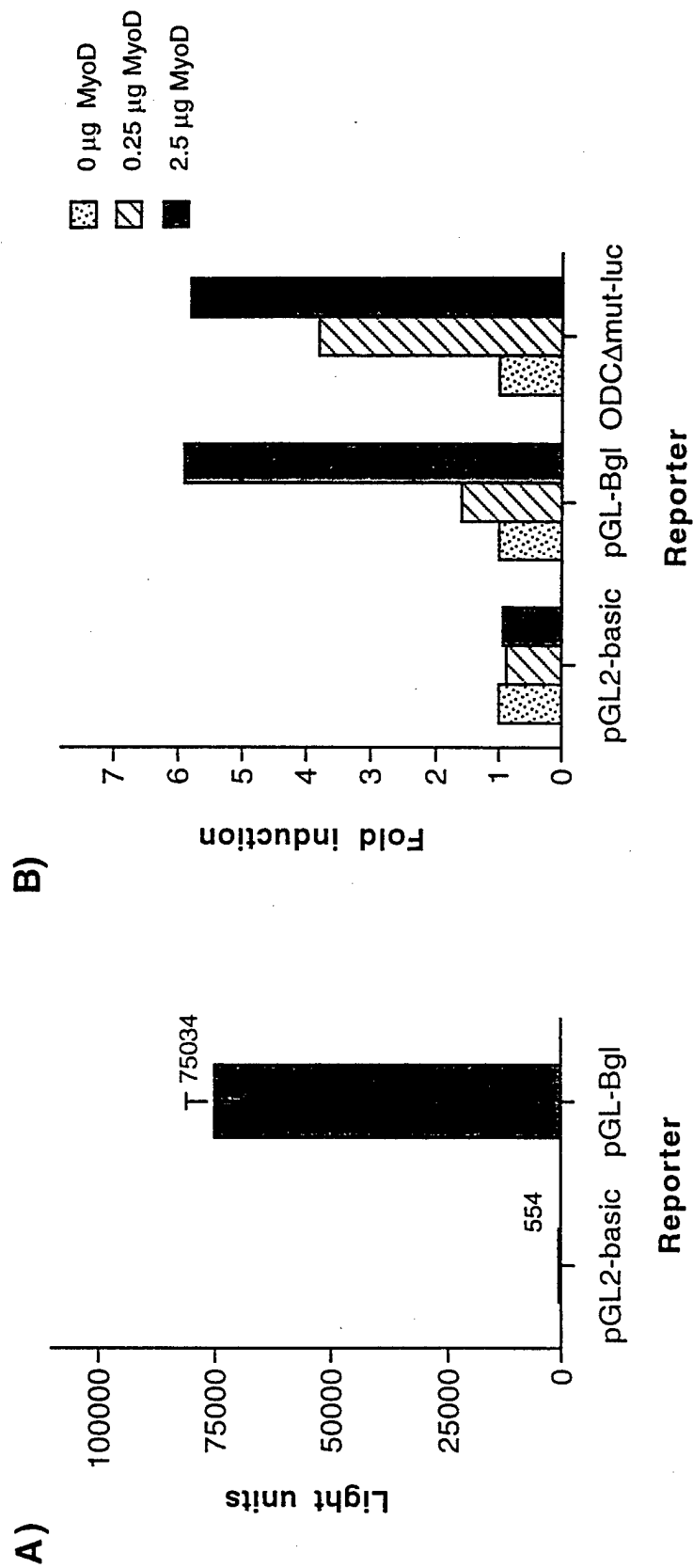
Fig. 5

```

agatctcggttcctcgatcccggcggttggttgtaaagtaggaacagtaatagcacctacctctagggccacggagaccg -695
aatgaaataatgcctgtcaagtcacttagtacacagtaagcactcagtaatgctaggttggttgattataataaatttt -615
tagcgaacggggaagaccaagcaccggttggtactgggttaggcgcgtagggcaaagatgtggagatgtcccggaggcg -535
cctaggggtatccgggcgaaaacccgagggccgaaggctgggaggaggcggagcgctcgggcaccgggcaccgggcgggagg -455
tgagccccctggaaaaggaggggactccgggcgcgttctcccagcagcgcggctcctctgttcaggggcgcgcccccttc -375
gccgcactttttctttgatttcgaaagcactcctctcctccacctagtctcctttcctgggttcaggagagttactgct -295
                                     E box
ttgcggggaaagaacaagacgccaggccggcggttagtccccgccccggggcggtgcagctggagcgtcaggggagtc -215
cgctcgccgcagccccagcgccgcgcgcgcccatccatcctagaaggacctggcggtgccggcgcccggagtgcccttt -135
                                     TBP
aaaaggcagcttattgtccggagggggcgggcgggggcgccgaccgcggtgagggccggccctccctctccctcc -55
                                     +1 ----- exon 1 ----->
ctctgtcccccgctcgctcgctggctagctcgctggctcgctcgccgctcggcGCACGCTCCGCCTCCGTCAGTTGGCT 26
CCGCTGTCGGGTTCGCGGGCGTGAGCGGCAGCCGGTCTGGACGCGCGGGCGGGGCTGGGGGCTGGGAGCGCGGCGCGCA 106
AGATCT 112

```

Fig. 6



Definition of regions of BIN1 required to inhibit MYC transactivation or to suppress malignant cell growth via MYC-dependent or MYC-independent mechanisms

Katherine Elliott^{1,2*}, Daitoku Sakamuro^{1*}, Wei Du^{1,3}, and George C. Prendergast¹

¹*Wistar Institute, 3601 Spruce Street, Philadelphia PA 19104;*

²*Cell and Molecular Biology and* ³*Biology Graduate Groups,*

University of Pennsylvania, Philadelphia PA 19104

Running title: BIN1 functional regions

*These authors contributed equally to this study

Corresponding author: G.C. Prendergast

Phone: (215) 898-3792

Facsimile: (215) 898-2205

March 7, 1997

*Oncogene,
submitted
under
revisions*

Abstract

BIN1 interacts with the N-terminal region of the MYC oncoprotein and has features of a tumor suppressor. BIN1 inhibits cell transformation by MYC but also by adenovirus E1A, and, as shown here, by mutant p53. In this report, we demonstrate that BIN1 inhibits MYC transactivation and we identify regions of BIN1 needed to suppress cell growth via both MYC-dependent and MYC-independent mechanisms. Similar to its requirement for inhibiting MYC transformation, BIN required its MYC-binding domain (MBD) to inhibit transactivation of ornithine decarboxylase, a physiological MYC target gene. Experiments in which BIN1 was fused to the DNA binding domain of the yeast transcription factor GAL4 suggested that BIN1 acts as an adaptor to recruit a repression function(s). Rat embryo fibroblast (REF) cotransformation and HepG2 tumor cell growth assays were used to define functionally important regions outside of the MBD. An N-terminal region termed BAR-C was crucial to inhibit MYC transformation but was also needed to efficiently inhibit E1A transformation. A second region located between BAR-C and the MBD, termed U1, was crucial for inhibiting E1A or mutant p53, but largely dispensible for inhibiting MYC. Finally, although dispensible elsewhere, BIN1's C-terminal SH3 domain was required to inhibit transformation mutant p53. In HepG2 cells, the MBD was essentially dispensible but BAR-C, U1, and SH3 were each important for efficient growth suppression. We concluded that BIN1 can inhibit the transactivating properties of MYC and can suppress cell growth through at least three mechanisms, two of which are MYC-independent.

Introduction

MYC is a central regulator of cell proliferation and apoptosis that is frequently activated in human malignancy (reviewed in Cole, 1986; Evan et al., 1995; Henriksson and Lüscher, 1996; Prendergast, 1997). In normal cells induced to divide, MYC levels rapidly increase and remain elevated, indicating it is required continuously for cell growth. Deregulated expression of MYC is sufficient to drive cells into the cell cycle and prevents cell cycle exit. Conversely, suppression of MYC blocks mitogenic signals and can contribute to terminal differentiation. MYC can also induce apoptosis, an activity which is manifested in normal cells where its expression is uncoupled from other cell cycle regulatory events. This feature is intriguing insofar as it may present an Achilles heel in tumor cells that contain deregulated MYC. The oncogenic properties of MYC are believed to be a consequence of its ability to act as a transcription factor. However, the exact mechanism underlying its action is not yet clear.

In an effort to address this issue, we previously identified a cellular polypeptide, BIN1, which interacts with the putative transcriptional regulatory domain of MYC (Sakamuro et al., 1996). The interaction depends upon the integrity of the so-called MYC boxes, two evolutionarily conserved segments which are necessary for both cell transformation and apoptosis. A role for BIN1 in controlling cell growth and cell cycle transit has been suggested by several observations. First, BIN1 inhibits cell transformation by MYC but also by adenovirus E1A (Sakamuro et al., 1996). Second, BIN1 is related to amphiphysin, a neuronal protein which is the autoimmune target of paraneoplastic disorders associated with breast and lung cancer (David et al., 1994; Dropcho, 1996), and RVS167, a negative regulator of the cell cycle in yeast (Bauer et al., 1993). Third, although widely expressed in normal cells, BIN is poorly expressed or undetectable in ~50% of carcinoma cell lines and primary breast carcinomas examined (Sakamuro et al., 1996). Fourth, deficits in expression are functionally significant, because ectopic BIN1 can inhibit the growth of tumor cells which lack endogenous expression (Sakamuro et al., 1996). Finally, the human *BIN1* gene has been mapped to chromosome

2q14 (Negorev et al., 1996), within a mid-2q region that is deleted in ~42% of metastatic prostate cancers (Cher et al., 1996), and at the syntenic murine locus, a site deleted in >90% of radiation-induced myeloid leukemias (Hayata et al., 1983).

We investigated the effects of BIN1 on MYC transactivation and defined the regions of BIN1 required to inhibit oncogene-mediated cell transformation or tumor cell growth. The data support a potential physiological link between BIN1 and MYC by showing that BIN1 can affect the transcriptional as well as the oncogenic properties of MYC. Furthermore, the results establish a broader role for BIN1 in cell growth regulation and delineate the regions needed to block cell growth through at least three mechanisms, only one of which is MYC-dependent.

Results

Construction and expression of BIN1 deletion mutants. To begin to define BIN1 functional regions, we generated a set of deletion mutants in p99f, a full-length BIN1 cDNA (Sakamuro et al., 1996). BAR-C and SH3 domain deletions encompass terminal regions of BIN1 that are structurally related to the neuronal protein amphiphysin and to the yeast cell cycle regulator RVS167. BAR-C represents the C-terminal half of the N-terminal BAR region, whose nomenclature reflects the homology with these proteins. The SH3 domain is located at the C-terminus, immediately adjacent to the MBD, and is dispensable for interaction with MYC (Sakamuro et al., 1996). Several deletions encompassed parts of the central region which is not conserved in amphiphysin or RVS167. These parts include the unique-1 (U1) region, a nuclear localization signal-like motif (NLS), the MBD, as initially defined (aa 270-389), or subsections of the MBD, including aa 270-288, aa 270-315 (also referred to as the unique-2 (U2) region), and aa 323-356. To confirm expression of mutant polypeptides, extracts from metabolically labeled COS cells transfected with vectors for each were processed for immunoprecipitation with 99DEFI, a mixture of four anti-BIN1 monoclonal antibodies (Wechsler-Reya et al., 1997a). The apparent and predicted MWs of the mutants did not coincide in

each case because of the presence of a determinant for aberrant gel mobility that maps to the MBD region (Sakamuro et al., 1996). However, each mutant was observed to accumulate as efficiently as full-length BIN1 (see Figure 1).

BIN1 inhibits transactivation of ornithine decarboxylase by MYC in an MBD-dependent manner. We previously showed that BIN1 interacted with MYC and inhibited its oncogenic activity (Sakamuro et al., 1996). The interaction requires the presence of Myc boxes 1 and 2, two N-terminal segments which are crucial for the biological functions of MYC (Henriksson and Lüscher, 1996; Prendergast, 1997). Because the Myc boxes are part of the putative transactivation domain (TAD) of MYC (Kato et al., 1990), we investigated the ability of BIN1 to affect MYC activation of ornithine carboxylase (ODC), a well-characterized physiological target gene (Bello-Fernandez et al., 1993; Wagner et al., 1993; Tobias et al., 1995; Packham and Cleveland, 1997) that has been implicated in cell transformation, tumorigenesis, and apoptosis (Auvinen et al., 1992; Moshier et al., 1993; Packham and Cleveland, 1994; Shantz and Pegg, 1994; Manni et al., 1995). The ODC reporter gene used, ODC Δ Luc, includes the 5' end of the murine ODC gene containing 5' flanking sequences, exon 1, intron 1, and exon 2; intron 1 contains the two E-box CACGTG sites that are recognized by the physiological MYC/MAX oligomer and that are required for MYC transactivation (Bello-Fernandez et al., 1993; Packham and Cleveland, 1997). NIH3T3 cells were used as a host for transient transfection of ODC Δ Luc, a c-MYC expression vector, and expression vectors for BIN1, a BIN1 mutant lacking the MYC-binding domain (BIN1 Δ MBD), or empty vector. As a positive control for suppression, we added trials including instead of BIN1 the retinoblastoma (Rb)-related protein p107, which has been reported to interact with the MYC TAD and inhibit its activity (Beijersbergen et al., 1994; Gu et al., 1994). Two days post-transfection, cells were harvested and cell lysates were prepared and assayed for luciferase activity.

1995). BIN1 and p107 each reversed this effect (see Figure 1A). The BIN1 inhibition was relieved

by deletion of the MBD, which is both sufficient to bind MYC and necessary to inhibit MYC transformation (Sakamuro et al., 1996). An augmentation of MYC transactivation by BIN1 Δ MBD was seen in some trials. To rule out that BIN1's inhibitory activity was cell type-dependent, we compared the effects of BIN1 and BIN1 Δ MBD in HeLa and NIH3T3 cells. We observed a similar MBD-dependent inhibition in each cell type (see Figure 1B). The inability of BIN1 Δ MBD to suppress MYC activity in these experiments was not due to polypeptide instability nor to general loss of function, because BIN1 Δ MBD accumulated similarly to wild-type BIN1 in transfected COS cells and was also capable of inhibiting E1A transformation ((Sakamuro et al., 1996); and see below). We concluded that BIN1 inhibited the E box-dependent transactivating activity of MYC in an MBD-dependent manner.

BIN1 recruits a repression activity that is sensitive to target gene context. We were interested in knowing whether BIN1 could inhibit MYC TAD activity outside of a target gene context, similar to p107 (Hoang et al., 1995; Smith-Sorensen et al., 1996). In addition, we wished to distinguish between the possibilities that BIN1 acted passively, by occluding coactivators, or actively, by delivering a repression function (either intrinsic to BIN1 or mediated by BIN1 interaction). To address the first issue, we tested whether BIN1 could inhibit the transcriptional activity of a GAL4 chimera, GAL4-MYC(1-262) (Kato et al., 1990), which includes all of MYC except for the b/HLH/LZ domain which mediates oligomerization with MAX and physiological DNA binding. To address the second issue, we generated chimeric DNA binding molecules that could deliver BIN1 to DNA in a MYC-independent fashion, to see if BIN1 had a repressive quality on its own.

HeLa cells were transfected with the GAL4 reporter gene GAL4-E1b-luc and equivalent amounts of expression vectors for GAL4-MYC(1-262) and either BIN1 or no insert. Cell lysates were prepared two days posttransfection and processed for luciferase activity. In HeLa cells, we observed that the activity of GAL4-MYC(1-262) was an average of 2.3-fold greater than unfused GAL0. Notably, BIN1 did not inhibit the activity of GAL4-MYC(1-262), but instead actually augmented it

approximately 60% (data not shown). Recently, it was reported that the relative transactivation activity of GAL4-MYC is as much as ~100-fold greater in U2OS osteosarcoma cells (Smith-Sorensen et al., 1996). Therefore, we conducted a similar set of experiments in U2OS cells using either ODC Δ Luc and wt MYC or GAL4-E1b-luc and GAL4-MYC(1-262). In these cells, GAL4-MYC(1-262) activity was very high but was readily inhibited by BIN1. However, we could not validate this effect as a *bona fide* MYC inhibitory activity, because in U2OS we were unable to demonstrate MYC transactivation of ODC, a physiological target gene. In addition, the inhibitory activity in U2OS was MBD-independent because BIN1 and BIN1 Δ MBD each suppressed GAL4-MYC(1-262) similarly (data not shown). Taken together, we interpreted the data to mean that BIN1 might affect transcription by two mechanisms, but that only one of these may be physiological relevant because it was MBD-dependent, linked to a validated MYC activation function, and sensitive to genetic context.

To investigate the basis for transcriptional inhibition, BIN1 was fused in frame to the DNA binding domain of the yeast transcription factor GAL4, generating GAL4-BIN1. To control for the potential of the MBD to introduce MYC or MYC-binding coactivators to a GAL4-BIN1 DNA binding complex, chimeras that lacked the MBD (GAL4-BIN1 Δ MBD) were also tested. HeLa cells were transfected with the reporter gene GAL4-E1b-luc and equivalent amounts of expression vectors for unfused GAL4 DNA binding domain (GAL0), GAL4-BIN1, or GAL4-BIN1 Δ MBD, and cell lysates were processed for luciferase activity as before. We observed that full-length BIN1 was essentially benign but that BIN1 Δ MBD was repressive, exhibiting a ~2.5-fold reduction in activity relative to GAL0 (see Figure 2A). This effect was specific because GAL4-BIN1 Δ MBD elicited $\leq 20\%$ change in the activity of pGL2-basic, a luciferase reporter gene lacking GAL4 sites (data not shown).

To distinguish between the possibilities that this repressive activity was intrinsic to BIN1 or was recruited through interactions with BIN1, we asked if the activity could be titered. One would predict that if the activity was intrinsic, BIN1 would not affect repression by GAL4-BIN1 Δ MBD when included in the transfected DNA. Alternately, if the activity was recruited to the reporter gene through

interactions with BIN1, one would predict that BIN1 would compete for the activity and therefore titrate the repressive effect. In this experiment, we found that BIN1 or BIN1 Δ MBD similarly relieved the relative inhibitory activity of GAL4-BIN1 Δ MBD (see Figure 2B). Thus, we interpreted the result to mean that BIN1 was not intrinsically repressive but instead acted as an adaptor for repressive functions. To map the region(s) of BIN1 required to recruit this function, we cotransfected HeLa cells with GAL4-BIN1 Δ MBD and various BIN1 deletion mutants and then measured the relative transcriptional activity. This experiment revealed that a crucial region was the so-called U1 region, a functionally undefined segment located in the midsection of BIN1 (see Figure 2C). We concluded that BIN1 functions as an adaptor for a repression activity and that the integrity of the U1 region is crucial for the effect.

BAR-C is required with the MBD to suppress MYC transformation. We previously showed that BIN1 suppresses MYC-dependent focus formation as measured by the RAS cooperation assay in primary rat embryo fibroblasts (REFs) (Land et al., 1983; Ruley, 1983). To map regions required for suppressing MYC transformation, REFs were transfected with expression vectors for MYC, activated RAS, and full-length or mutant BIN1, and transformed cell foci were scored two weeks later. Consistent with previous results, wild-type BIN1 suppressed focus formation by MYC ~6-fold relative to the empty vector control. Most of the deletion mutants inhibited focus formation as efficiently as wt BIN1. Notably, the NLS-like motif and the SH3 domain were each dispensable for inhibition. In contrast, deletion of either BAR-C or aa 323-356, a central MBD segment, relieved inhibition. The MBD was originally defined as a region located between aa 270-389. However, the N-terminal segment located between aa 270-315 was dispensable. Thus, the critical part of the MBD was located to a 66 residue segment between aa 323-389. Because it was dispensable for inhibiting MYC, we renamed the aa 270-315 region the unique-2 (U2) region (since like U1 it is not conserved in amphiphysin or RVS167). The inability of BIN1 Δ 323-356 or BIN1 Δ BAR-C to inhibit MYC was not due to instability of the mutant polypeptides, because each accumulated stably in COS cells similar to wt BIN1 (see Figure 4). Moreover, these mutants did not suffer a general loss of activity (e.g. due

to protein misfolding), because they were able to suppress MYC-independent transformation (see below). However, consistent a specific lack of activity, exogenous BIN1 Δ BAR-C and BIN1 Δ 323-356 messages could be detected by Northern analysis of RNA from pools of foci that harvested and cultured (data not shown). We concluded that BAR-C along with the MBD was required to suppress MYC transformation.

U1 is crucial and BAR-C is important for suppressing E1A transformation; U1 and SH3 are both crucial to suppress mutant p53 transformation. We previously showed that BIN1 could inhibit E1A-dependent transformation of REFs in a manner that was MBD-independent (Sakamuro et al., 1996). This result implied that, in addition to its ability to inhibit MYC-dependent growth, BIN1 could also inhibit growth via a MYC-independent mechanism(s). To further investigate the scope of BIN1's inhibitory properties, we tested whether BIN1 could also inhibit REF cotransformation induced by oncogenic RAS and a dominant inhibitory mutant of p53. We then proceeded to assay various BIN1 deletion mutants to determine whether there was any overlap in the regions required to inhibit MYC-independent and MYC-dependent cell transformation.

BIN1 was found to inhibit transformation by mutant p53, as well as by MYC and E1A, but the domain requirements for each oncogene were different (see Figure 5). Inhibition of focus formation by mutant p53 was less potent than MYC or E1A, with a 3-fold inhibition of mutant p53 seen under the same conditions relative to a 6- to 7-fold inhibition of the other oncoproteins. While necessary to inhibit MYC, the MBD was dispensable to inhibit either mutant p53 or E1A, indicating a MYC-independent mechanism of action. As before, the NLS-like motif was also dispensable. In strong contrast, U1 deletion relieved inhibition of either mutant p53 or E1A and deletion of SH3 abolished inhibition of mutant p53. BAR-C was required for efficient inhibition of E1A, but the effect of BAR-C deletion was more subtle compared to the effect of U1 deletion. As before, the lack of activity of BIN1 Δ U1 and BIN1 Δ SH3 could not be explained by polypeptide instability or a general loss of activity, since each mutant was able to suppress MYC transformation. However, consistent with a

selective lack of activity, exogenous BIN1 Δ U1 or BIN1 Δ U1 and BIN1 Δ SH3 messages were detected by Northern analysis of RNA from pools of foci cultured from cells transfected with E1A or mutant p53, respectively (data not shown). Thus, the domains required to inhibit E1A and mutant p53 were overlapping, but distinct, and in each case different from those required to block MYC. We concluded that BIN1 could inhibit MYC-independent transformation through two mechanisms that required U1 or the SH3 domain, respectively.

BAR-C, U1, and SH3 are each important for efficient suppression of tumor cell growth. We previously showed that BIN1 can suppress the proliferation of HepG2 hepatocarcinoma cells, which lack endogenous BIN1 expression, using a G418-resistant colony formation assay (Sakamuro et al., 1996). This assay was used to assess the ability of the BIN1 deletion mutants to suppress tumor cell growth. HepG2 cells were transfected with neomycin resistance gene-marked vectors for full-length or mutant BIN1 and G418-resistant colonies were scored approximately 3 weeks later (see Figure 6). We observed that deletion of NLS or the MBD had no significant effect. However, an overlap with MYC requirements for inhibition existed insofar as BAR-C deletion provided the most-potent, though partial, relief of BIN1 inhibition. Deletion of U1 and SH3 also partially relieved inhibition. The observation that none of these domains were completely dispensable, as was the case in REF transformation, indicated that each contributed to efficient inhibition in tumor cells. We concluded that BAR-C, U1, and SH3 were each important for suppression of tumor cell growth by BIN1.

Discussion

In this study, we showed that BIN1 can inhibit the transcriptional as well as the oncogenic properties of MYC. We also showed that BIN1 can inhibit cell transformation by mutant p53 as well as by adenovirus E1A, through MYC-independent mechanisms. Finally, we identified regions outside of the MBD that are crucial to inhibit cell transformation or malignant cell growth, through MYC-

dependent or MYC-independent mechanisms. The functional regions of BIN1 mapped in this study are summarized in Figure 7. Our findings support the hypothesis of a physiological link between BIN1 and MYC and suggest that BIN1 may have a broad role in regulating cell growth by diverse mechanisms.

BIN1 and transcription. We found that BIN1 could inhibit the ability of MYC to transactivate ODC, a likely target gene, in a manner that required the MBD, a domain that is sufficient for MYC interaction (Sakamuro et al., 1996). Analysis of this activity indicated that it was not intrinsic but instead reflected an adaptor function which could recruit a repression activity(s). Such an adaptor function fits well with the function of BIN1 that is hinted at by the presence of an SH3 domain, a feature of many cytosolic adaptors. The BAR domain was implicated in the recruitment of a repressor function, suggesting a transcriptional role for this region. We showed here that BIN1 can inhibit MYC activation of an E box-dependent gene. In the accompanying paper, BIN1 was also shown to inhibit MYC activation of *cdc2*, a putative E box-independent target gene (Born et al., 1997). Thus, the ability of BIN1 to interact with the N-terminal TAD domain of MYC may allow it to inhibit transactivation of both E box-dependent and E box-independent classes of target genes.

We observed that BIN1's ability to inhibit the MYC TAD varied with genetic context. These observations are consistent with previous observations that BIN1 could not inhibit MYC transactivation of an artificial promoter containing multimerized MYC/MAX binding sites (Sakamuro et al., 1996). Taken together, the data suggested that MYC target genes such as ODC provide a special context that reveals BIN1 activity. In light of this aspect, it is intriguing to note that the current set of genes most widely considered to be physiological MYC target genes, including ODC and CDC25, each have the MYC/MAX binding sites located in the first or second intron rather than a more "traditional" location in the 5' flanking region (Henriksson and Lüscher, 1996; Prendergast, 1997). BIN1's need for a *bona fide* genetic context, a feature that differs from p107, provides additional support for the possibility that BIN1 and MYC may interact physiologically. While promoter

organization may partly explain the selectivity of BIN1, a nuclear adaptor role, perhaps operating to integrate transcriptional and cell cycle functions, is also likely to be relevant. BIN1 lacks an intrinsic repressive quality so it may only inhibit MYC activation if it can interact with repression functions. If the availability, repressive potential, or BIN1 interactive capability of these functions depend upon additional factors, as one might anticipate, then one would expect BIN1 to be insufficient to inhibit MYC transactivation in all cases. Thus, given biochemical and architectural differences among promoters, BIN1 may be able to exert a repressive effect at some but not all MYC target loci. This possibility presents an opportunity for combinatorial regulation of sets of target genes that may distinguish the different biological actions of MYC. Tests of such a model will become possible as greater numbers of candidate MYC target genes are accumulated.

BIN1 and cell growth regulation. We found that BIN1 required both MBD and BAR-C function to suppress MYC transformation. A 45 aa segment (aa 270-315) located at the N-terminal end of the previously defined MBD was shown to be dispensable, indicating that the critical MBD region is located immediately upstream of the SH3 domain. Characterization of the human BIN1 gene structure has indicated that this region is encoded by two exons, with the more 5' exon closely overlapping the aa 323-358 deletion which relieves MYC suppression (Wechsler-Reya et al., 1997b). Interestingly, this exon is alternately spliced in many cells. Thus, a segment of the MBD defined here to be crucial for suppressing the oncogenic activity of MYC is regulated *in vivo* by splicing. The nature of the BAR-C function required is unclear. Given that gene activation by MYC is widely believed to be necessary for cell transformation (Henriksson and Lüscher, 1996; Prendergast, 1997), it is tempting to speculate that this function may overlap or interact with the one in U1 implicated in transcriptional repressor recruitment. Further analysis will be needed to determine if this is the case.

We extended the scope of BIN1's growth inhibitory action by showing that BIN1 can suppress transformation by mutant p53 as well as by E1A. Notably, these capabilities depended upon regions that were dispensable for suppressing MYC activities. Inhibition of MYC-dependent growth depended

upon the MBD and BAR-C, while inhibition of MYC-independent growth inhibition depended upon U1 and SH3. Interestingly, there was not an exact overlap in the regions required to suppress either E1A or mutant p53. E1A suppression depended mainly upon U1; BAR-C was also important for efficient suppression, but it was clearly secondary to U1. Mutant p53 suppression similarly depended upon U1, but there was also a unique requirement for the SH3 domain. We inferred from these different requirements that there are at least two mechanisms by which BIN1 can block MYC-independent growth.

Three regions shown to be important for suppressing oncogene-mediated REF transformation, BAR-C, SH3, and U1, were also demonstrated to be critical for BIN1 to exert a tumor suppressor-like activity in HepG2 cells. The finding that no one domain was crucial for growth inhibitory activity, as had been the case in REF assays, is perhaps unsurprising given that many regulatory pathways are altered in tumor cells. The need for each of the domains identified in the REF assay suggested that suppression of both MYC-dependent and MYC-independent pathways is needed to efficiently block tumor cell growth. The reason that the MBD was dispensable in HepG2 was unclear, especially since these cells overexpress MYC (D.S., unpublished observations), although one might interpret the finding to imply that MYC-independent growth pathways are dominant.

Non-MBD regions of BIN1. Based on its sequence, which is nearly identical to "classical" NLS elements, we had tentatively assigned an NLS-like motif in the central region of BIN1 a role in nuclear localization (Sakamuro et al., 1996). However, several lines of evidence have called this into question. First, while BIN1 localizes to the nucleus of growing cells (Sakamuro et al., 1996; Wechsler-Reya et al., 1997a), indirect immunofluorescence experiments have indicated that BIN1 Δ NLS can also localize to the nucleus as well as BIN1 or the other deletion mutants used in this study (K.E. and G.C.P., unpublished results). Second, the NLS-like sequences in BIN1 are encoded by an exon in the human gene which is subjected to alternate splicing (Wechsler-Reya et al., 1997b), and forms of BIN1 derived from RNAs lacking this exon can still localize to the nucleus (R. Wechsler-

Reya and G.C.P., unpublished observations). Thus, although from its sequence it may be sufficient for nuclear localization, the NLS motif in BIN1 is clearly not necessary, since other regions of BIN1 appear to substitute efficiently.

Important roles for the BAR-C, U1, and SH3 regions were revealed by this study. BAR-C is a highly charged region of 84 aa predicted to be largely α helical in character (K.E., unpublished observations). It encompasses three exons in the human gene (Wechsler-Reya et al., 1997b) so may contain functionally smaller units. Consistent with the possibility that it encodes at least one domain, we have found that a BAR-C expression cassette can specifically inhibit E1A transformation ~2-fold (G.C.P., unpublished observations). U1 is a 28 aa region, not conserved in amphiphysin or RVS167, that is located between the ends of the BAR domain and the NLS-like motif in BIN1. This region is encoded by a single exon in the human gene (Wechsler-Reya et al., 1997b). While too small to constitute a domain itself, it may contain or contribute elements that are important for intra- or intermolecular interactions. The SH3 domain has a unique loop 4 insert that may affect binding specificity (Koyama et al., 1993; Sparks et al., 1996), although its nuclear interaction targets in are not yet known. The requirement for suppressing mutant p53 is intriguing given direct interactions that have been seen *in vitro* between the BIN1 SH3 domain and p53 (D.S, unpublished observations), possibly through a polyproline (PP) region in the N-terminus that is required for efficient growth suppression (Walker and Levine, 1996) and that we have shown is a critical apoptotic effector domain (Sakamuro et al., 1997). This link, while speculative, might be germane to gain-of-function effects of mutant p53 in cell transformation that are derived from activities other than oligomerization (Dittmer et al., 1993). In future work, it will be important to focus on the BAR-C, U1, and SH3 regions as a means to gaining insight into the functions of BIN1 in cell growth control.

Materials and Methods

Plasmid constructions. The following plasmids have been described. CMV-BIN1 and CMV-BIN1 Δ MBD express a full-length BIN1 cDNA or a deletion mutant lacking the MYC-binding domain (MBD) (Sakamuro et al., 1996). LTR Hm contains a Moloney long terminal repeat-driven normal human *c-myc* gene (Kelekar and Cole, 1986); p1A/neo contains the 5' end of the adenovirus type 5 genome including the E1A region (Maruyama et al., 1987); LTR p53ts encodes a temperature-sensitive dominant inhibitory mutant of murine p53 (Michalovitz et al., 1990); and pT22 contains an activated H-ras gene (Land et al., 1983). ODC Δ Luc, whose construction has been described (Packham and Cleveland, 1997), contains a luciferase expression cassette downstream of a ~2.5 kb genomic DNA fragment that includes 5' flanking sequences, exon 1, intron 1, and exon 2 from the murine ODC gene. Gal5-SV40-luc is a GAL4 reporter; GAL0 expresses the DNA binding domain of GAL4 (aa 1-143); and GAL4-MYC(1-262) encodes a chimeric polypeptide which includes all of human MYC except its DNA binding b/HLH/LZ region (Kato et al., 1990). CMV-p107 is a derivative of the cytomegalovirus enhancer/promoter-containing vector pcDNA3 (Invitrogen) containing human p107 cDNA (Zhu et al., 1993).

BIN1 deletion mutants and GAL4 fusion genes were subcloned for expression in pcDNA3, the same vector used to express BIN1 and BIN1 Δ MBD. BIN1 Δ BAR-C was constructed by dropping an internal Afl III restriction fragment from CMV-BIN1, resulting in a deletion of aa 125-207 from the BAR domain (Sakamuro et al., 1996). The remaining mutants were generated by standard PCR methodology using the oligonucleotide primers 995'(Bam), 993'SH3(Xho) (Sakamuro et al., 1996) and others whose sequence is derived from the BIN1 cDNA sequence (Genbank accession number U68485). The integrity of PCR-generated fragments was verified by DNA sequencing. Oligonucleotides and details for each construction, omitted to conserve space, are available from G.C.P. BIN1 Δ U1 lacks aa 224-248; BIN1 Δ NLS, aa 251-269; BIN1 Δ SH3, aa 384-451; the other mutants lack the residues indicated. GAL4 fusions were generated in two steps by first subcloning the

143 aa DNA binding domain from GAL0 into pcDNA3 and then ligating in-frame full-length BIN1, BIN1 Δ MBD, or MBD (aa 270-383) sequences downstream.

Cell culture. COS, HeLa, HepG2 cells from the ATCC were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum (Atlantic) and 50 U/ml penicillin and streptomycin (Fisher). NIH3T3 cells were cultured in DMEM supplemented with 10% calf serum (Gibco) and antibiotics (transfections were performed in media containing 10% fetal calf serum). Rat embryo fibroblasts (REFs) were obtained from Whittaker Bioproducts and cultured as described (Prendergast et al., 1992). For transformation assays, secondary passage REFs seeded in 10 cm dishes were transfected overnight by a calcium phosphate coprecipitation method (Chen and Okayama, 1987) with 5 μ g each of oncogenic RAS plus MYC, E1A, or mutant p53 expression plasmids and 10 μ g of BIN1 plasmid or empty vector. Cells were fed and the next day passaged into 1 15 cm dish (MYC transfections) or 3 10 cm dishes (E1A or mutant p53 transfections). Foci were scored by methanol fixation and crystal violet staining 12-16 days later. Colony formation assays in HepG2 cells were performed by seeding $\sim 3 \times 10^5$ cells in 6 cm dishes and transfecting the next day with 2 μ g plasmid DNA using Lipofectamine (Gibco/BRL). Cells were passaged 48 hr after transfection at a 1:10 ratio into 6 cm dishes containing media with ~ 0.6 mg/ml G418 and cell colonies were scored by crystal violet staining ~ 3 weeks later.

Immunoprecipitation. COS cells were metabolically labeled for 2 hr in DMEM media lacking methionine and cysteine (Gibco) with 100 μ Ci/ml EXPRESS labeling reagent (NEN) and cell extracts were prepared with NP40 buffer containing the protease inhibitors leupeptin, aprotinin, phenylmethylsulfonyl fluoride, and antipain (Harlow and Lane, 1988). Extracts were centrifuged at 20000 g for 15 min at 4°C before use. Extracts were precleared by a 1 hr treatment with prebleed sera or normal mouse IgG and 20 μ l of a 1:1 slurry of protein G Sepharose beads at 4°C on a nutator (Pharmacia). A mixture of BIN1 monoclonal antibodies was used for immunoprecipitation (50 μ l each 99D,E,F,I) (Wechsler-Reya et al., 1997a). Immunoprecipitation proceeded 1 hr at 4°C followed by

the addition of protein G beads and a further 30 min incubation. Beads were collected by brief centrifugation, washed four times with buffer, boiled in SDS gel loading buffer, fractionated on 10% gels, and fluorographed.

Transactivation assays. NIH3T3 and HeLa cells seeded into 6 well dishes were transfected in duplicate wells as described above with 1.5 μ g ODC Δ S-luc, 3 μ g LTR-Hm, 1.5 μ g vectors indicated, and 0.5 μ g CMV- β gal (to normalize for transfection efficiency). pcDNA3 was added to bring the total DNA per each 2 well transfection to 6.5 μ g DNA. The experiment shown in Figure 1B was similar except that a 1:4:6.5 ratio of ODC Δ S-luc:LTR-Hm:BIN1 vectors was used. For the experiments using GAL4 fusions, HeLa cells seeded similarly were transfected with 2 μ g Gal5SV40-luc reporter, 4 μ g of the indicated chimeric gene, and 1 μ g CMV- β gal. Two days post-transfection, cell extracts were prepared and analyzed for luciferase and β galactosidase activity using a commercial kit (Promega), following the protocol provided by the vendor.

Northern analysis. Total cytoplasmic RNA was isolated as described (Prendergast and Cole, 1989) from pools of G418-resistant HepG2 cell colonies or REF foci. RNAs were fractionated on formaldehyde gels and hybridized to 32 P-labeled Bin1 cDNA probes (Church and Gilbert, 1984).

Acknowledgments

We thank Roberto Buccafusca for technical assistance, Chi Dang for GAL₅-SV40-luc, GAL0, and GAL4-MYC(1-262), and John Cleveland for ODCALUC. For discussion and critical comments, we thank Shelley Berger, Frank Rauscher III, and members of our laboratory. This work was supported by grants CN-160 from the ACS and DAMD17-96-1-6324 from the US Army Breast Cancer Research Program (G.C.P.). K.E. was supported by an NIH Training Grant. D.S. is the recipient of the 1997 Christopher Davis Memorial Fellowship. G.C.P. is the recipient of an American Cancer Society Junior Faculty Award and is a Pew Scholar in the Biomedical Sciences.

References

- Auvinen, M., Passinen, A., Andersson, L.C. and Holttä, E. (1992). *Nature*, **360**, 355-358.
- Bauer, F., Urdaci, M., Aigle, M. and Crouzet, M. (1993). *Mol. Cell. Biol.*, **13**, 5070-5084.
- Beijersbergen, R.L., Hijmans, E.M., Zhu, L. and Bernards, R. (1994). *EMBO J.*, **13**, 4080-4086.
- Bello-Fernandez, C., Packham, G. and Cleveland, J.L. (1993). *Proc. Natl. Acad. Sci. USA*, **90**, 7804-8.
- Born, T.L., Sakamuro, D., Prendergast, G.C. and Feramisco, J.R. (1997). *Oncogene*, submitted.
- Chen, C. and Okayama, H. (1987). *Mol. Cell. Biol.*, **7**, 2745-2752.
- Cher, M.L., Bova, G.S., Moore, D.H., Small, E.J., Carroll, P.R., Pin, S.S., Epstein, J.I., Isaacs, W.B. and Jensen, R.H. (1996). *Canc. Res.*, **56**, 3091-3102.
- Church, G.M. and Gilbert, W. (1984). *Proc. Natl. Acad. Sci. USA*, **81**, 1991-1995.
- Cole, M.D. (1986). *Ann. Rev. Genet.*, **20**, 361-384.
- David, C., Solimena, M. and De Camilli, P. (1994). *FEBS Lett.*, **351**, 73-79.
- Dittmer, D., Pati, S., Zambetti, G., Chu, S., Teresky, A.K., Moore, M., Finlay, C. and Levine, A.J. (1993). *Nat. Genet.*, **4**, 42-6.
- Dropcho, E.J. (1996). *Ann. Neurol.*, **39**, 659-667.
- Evan, G.I., Brown, L., Whyte, M. and Harrington, E. (1995). *Curr. Biol.*, **7**, 825-834.
- Gu, W., Bhatia, K., Magrath, I.T., Dang, C.V. and DallaFavera, R. (1994). *Science*, **264**, 251-254.
- Harlow, E. and Lane, D. (1988). Antibodies: A Laboratory Manual. Cold Spring Harbor NY, Cold Spring Harbor Laboratory Press.

- Hayata, I., Seki, M., Yoshida, K., Hirashima, K., Sado, T., Yamagiwa, J. and Ishihara, T. (1983). *Cancer Res.*, **43**, 367-373.
- Henriksson, M. and Lüscher, B. (1996). *Adv. Canc. Res.*, **68**, 109-182.
- Hoang, A.T., Lutterbach, B., Lewis, B.C., Yano, T., Chou, T.-Y., Barrett, J.F., Raffeld, M., Hann, S.R. and Dang, C.V. (1995). *Mol. Cell. Biol.*, **15**, 4031-4042.
- Kato, G.J., Barrett, J., Villa-Garcia, M. and Dang, C.V. (1990). *Mol. Cell. Biol.*, **10**, 5914-5920.
- Kelekar, A. and Cole, M. (1986). *Mol. Cell. Biol.*, **6**, 7-14.
- Koyama, S., Yu, H., Dalgarno, D.C., Shin, T.B., Zydowsky, L.D. and Schreiber, S.L. (1993). *Cell*, **72**, 945-952.
- Land, H., Parada, L.F. and Weinberg, R.A. (1983). *Nature*, **304**, 596-602.
- Manni, A., Wechter, R., Wei, L., Heitjan, D. and Demers, L. (1995). *J. Cell. Physiol.*, **163**, 129-36.
- Maruyama, K., Schiavi, S.C., Huse, W., Johnson, G.L. and Ruley, H.E. (1987). *Oncogene*, **1**, 361-7.
- Michalovitz, D., Halevy, O. and Oren, M. (1990). *Cell*, **62**, 671-681.
- Moshier, J.A., Dosesu, J., Skunca, M. and Luk, G.D. (1993). *Canc. Res.*, **53**, 2618-2622.
- Negorev, D., Reithman, H., Wechsler-Reya, R., Sakamuro, D., Prendergast, G.C. and Simon, D. (1996). *Genomics*, **33**, 329-331.
- Packham, G. and Cleveland, J.L. (1994). *Mol. Cell. Biol.*, **14**, 5741-7.
- Packham, G. and Cleveland, J.L. (1997). *Oncogene*, submitted.
- Prendergast, G.C. (1997). Myc function. Oncogenes as Transcriptional Regulators. Boston, Birkhauser Verlag.

- Prendergast, G.C. and Cole, M.D. (1989). *Mol. Cell. Biol.*, **9**, 124-134.
- Prendergast, G.C., Hopewell, R., Gorham, B. and Ziff, E.B. (1992). *Genes Dev.*, **6**, 2429-2439.
- Ruley, H.E. (1983). *Nature*, **304**, 602-606.
- Sakamuro, D., Elliott, K., Wechsler-Reya, R. and Prendergast, G.C. (1996). *Nature Genet.*, **14**, 69-77.
- Sakamuro, D., Sabbatini, P., White, E. and Prendergast, G.C. (1997). *EMBO J.*, submitted.
- Shantz, L.M. and Pegg, A.E. (1994). *Canc. Res.*, **54**, 2313-2316.
- Smith-Sorensen, B., Hijmans, E.M., Beijersbergen, R.L. and Bernards, R. (1996). *J. Biol. Chem.*, **271**, 5513-5518.
- Sparks, A.B., Hoffman, N.G., McConnell, S.J., Fowlkes, D.M. and Kay, B.K. (1996). *Nat. Biotech.*, **14**, 741-744.
- Tobias, K.E., Shor, J. and Kahana, C. (1995). *Oncogene*, **11**, 1721-7.
- Wagner, A.J., Meyers, C., Laimins, L.A. and Hay, N. (1993). *Cell Growth Diff.*, **4**, 879-883.
- Walker, K.K. and Levine, A.J. (1996). *Proc. Natl. Acad. Sci. USA*, **93**, 15335-15340.
- Wechsler-Reya, R., Elliott, K., Herlyn, M. and Prendergast, G.C. (1997a). *Cancer Res.*, submitted.
- Wechsler-Reya, R., Sakamuro, D., Zhang, J. and Prendergast, G.C. (1997b). *Proc. Natl. Acad. Sci. USA*, submitted.
- Zhu, L., van den Heuvel, S., Helin, K., Fattaey, A., Ewen, M., Livingston, D., Dyson, N. and Harlow, E. (1993). *Genes Dev.*, **7**, 1111-1125.

Figure Legends

Figure 1. Structure and expression of BIN1 deletion mutants. COS cells were transfected with the BIN1 expression vectors indicated and metabolically labeled with ^{35}S -methionine/cysteine two days later. Cell extracts were prepared and subjected to immunoprecipitation with a mixture of BIN1 monoclonal antibodies. Immunoprecipitates were processed by SDS-PAGE and fluorography.

Figure 2. BIN1 inhibits MYC transactivation of ornithine carboxylase in an MBD-dependent manner. (A.) MBD-dependent inhibition of MYC transactivation. NIH3T3 cells were transfected with a 1:2:1 ratio of ODC Δ S-luc luciferase reporter:MYC vector:plasmids indicated. Cell extracts were prepared and processed for reporter activity two days post-transfection. (B.) BIN1 inhibition is independent of cell type. NIH3T3 or HeLa cells were transfected with a 1:4:6.5 ratio of ODC Δ S-luc, MYC, and the plasmids indicated and processed as before.

Figure 3. BIN1 has an intrinsic transcriptional repression activity that is sensitive to genetic context. (A.) Intrinsic repressive quality of BIN1. HeLa cells were transfected with a 2:1 ratio of the indicated GAL4 fusion vector and GAL4 luciferase reporter and cell extracts were processed for luciferase activity as above. (B.) The repressive activity of BIN can be titrated. HeLa cells were transfected with a 2:2:1 ratio of vectors for GAL4-BIN Δ MBD, BIN1 or BIN1 Δ MBD, and GAL4 luciferase reporter and cell extracts were processed for luciferase activity as before. (C.) U1 is critical for titration of BIN1 repression activity. HeLa cells were transfected with a 2:3:1 ratio of vectors for GAL4-BIN Δ MBD, BIN1 or BIN1 Δ MBD, and GAL4 luciferase reporter and cell extracts were processed for luciferase activity as before. In each experiment, the activation activity is represented as a percentage of GAL0 activity.

Figure 4. BAR-C and a region of the MBD are required to inhibit MYC transformation. REFs were transfected with a 1:1:2 ratio of expression vectors for oncogenic RAS, deregulated human c-MYC, and the BIN1 mutants indicated. Transformed cell foci were scored 12-14 days later. At least three trials were performed with each mutant. The data are presented as a percentage of the foci formed by oncogenes plus empty vector.

Figure 5. U1 or U1 and SH3 are required to suppress transformation by E1A or mutant p53, respectively. (A.) REF foci assay. REFs were transfected with a 1:1:2 ratio of expression vectors for oncogenic RAS, adenovirus E1A or a dominant inhibitory p53 mutant, and the BIN1 mutants indicated. Transformed cell foci were scored 12-16 days later. For each trial, foci were scored in triplicate by passaging cells into three dishes after transfection. At least two trials were performed with each mutant, except the U1 or SH3 deletions, which were tested 5 times each.

Figure 6. BAR-C, U1, SH3 are each important for suppressing tumor cell growth. HepG2 cells were transfected with equivalent amounts of the indicated neomycin (neo^r)-resistance gene marked expression vectors. G418-resistant cell colonies were scored 2-3 weeks later by methanol fixation and crystal violet staining. The efficiency of colony formation for each vector is presented as a percentage of empty vector.

Figure 7. Regions of BIN1 required for its various activities. The hatched bars indicate a partial requirement. MYC interaction data derived from Sakamuro et al., 1996.

Fig. 1

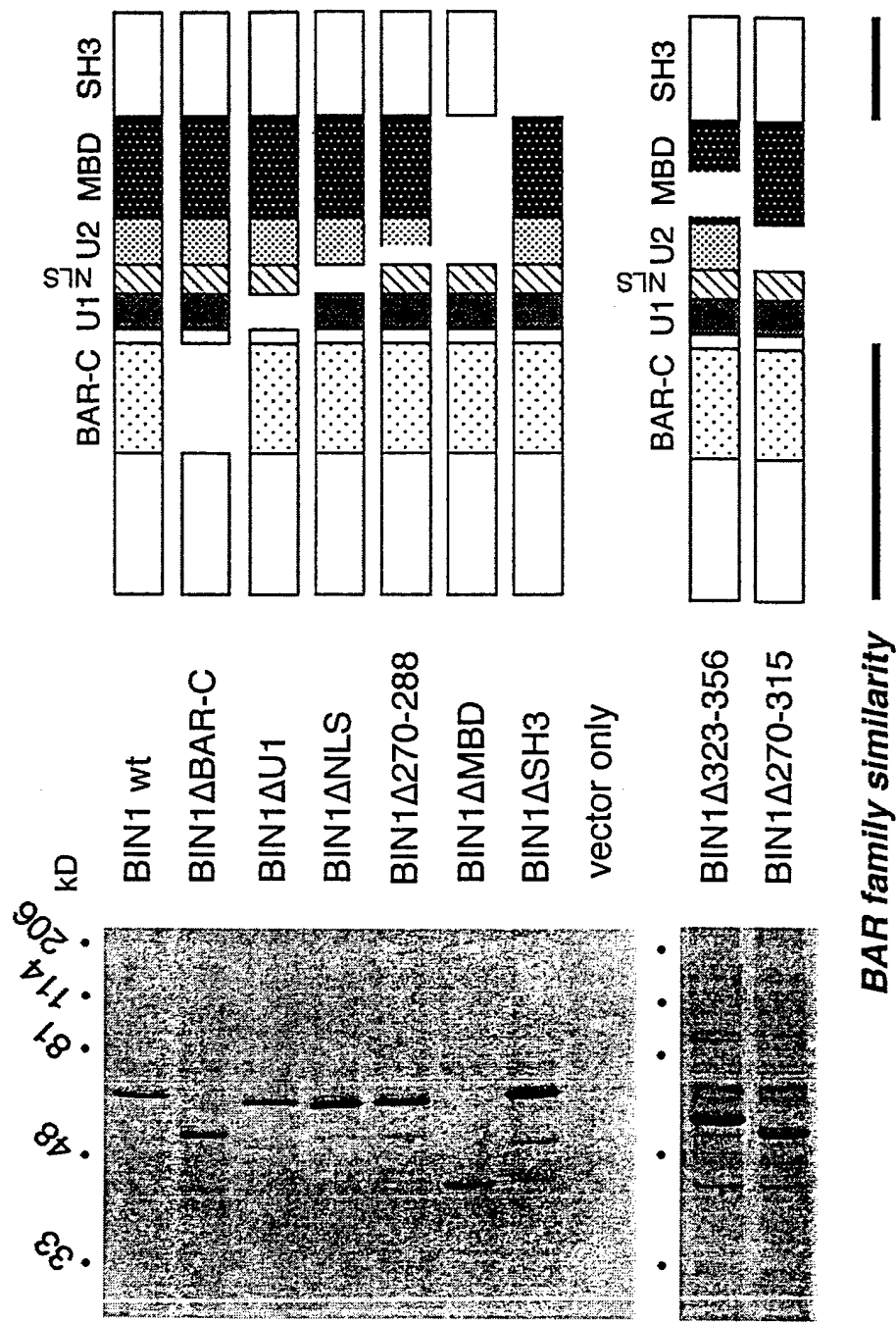


Fig. 2

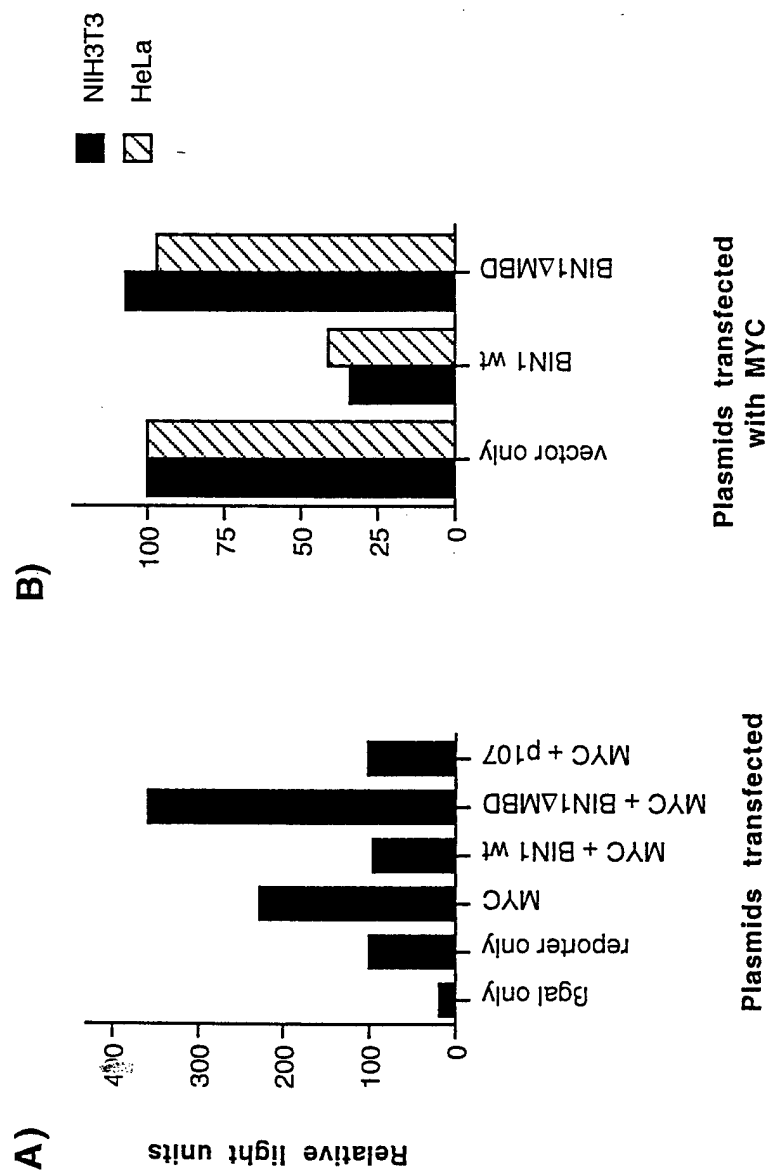


Fig. 3

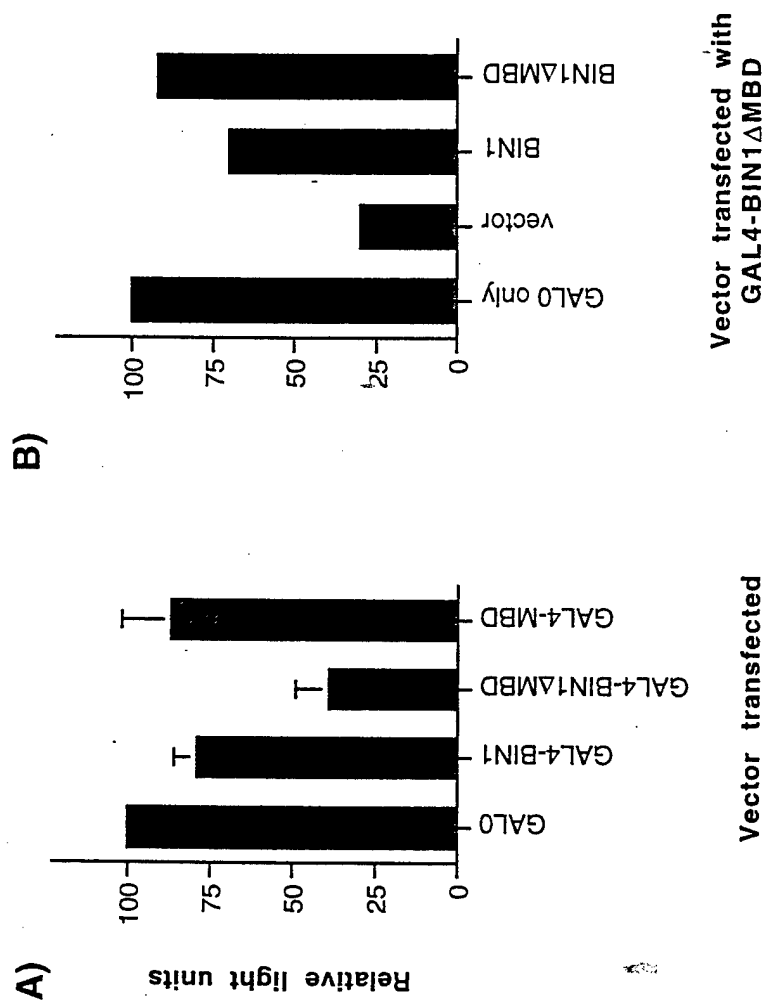


Fig. 4

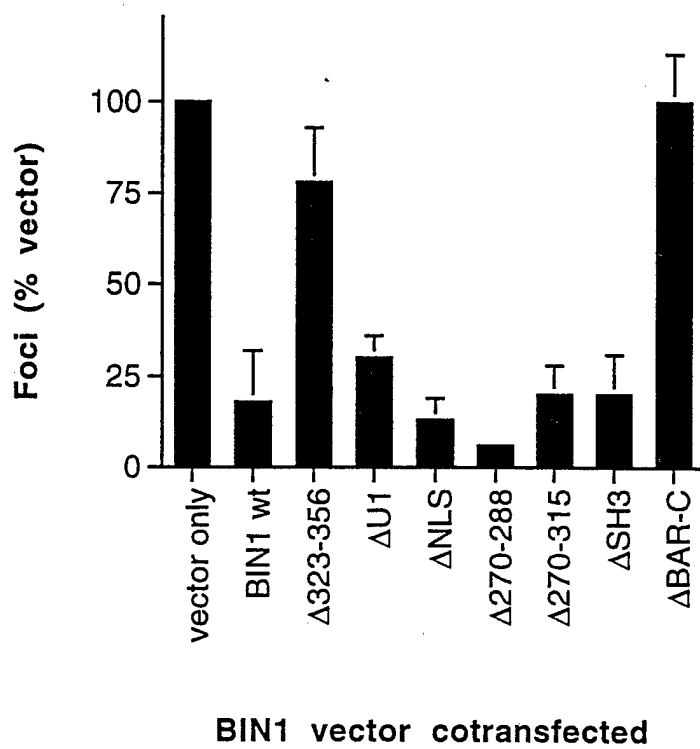


Fig. 5

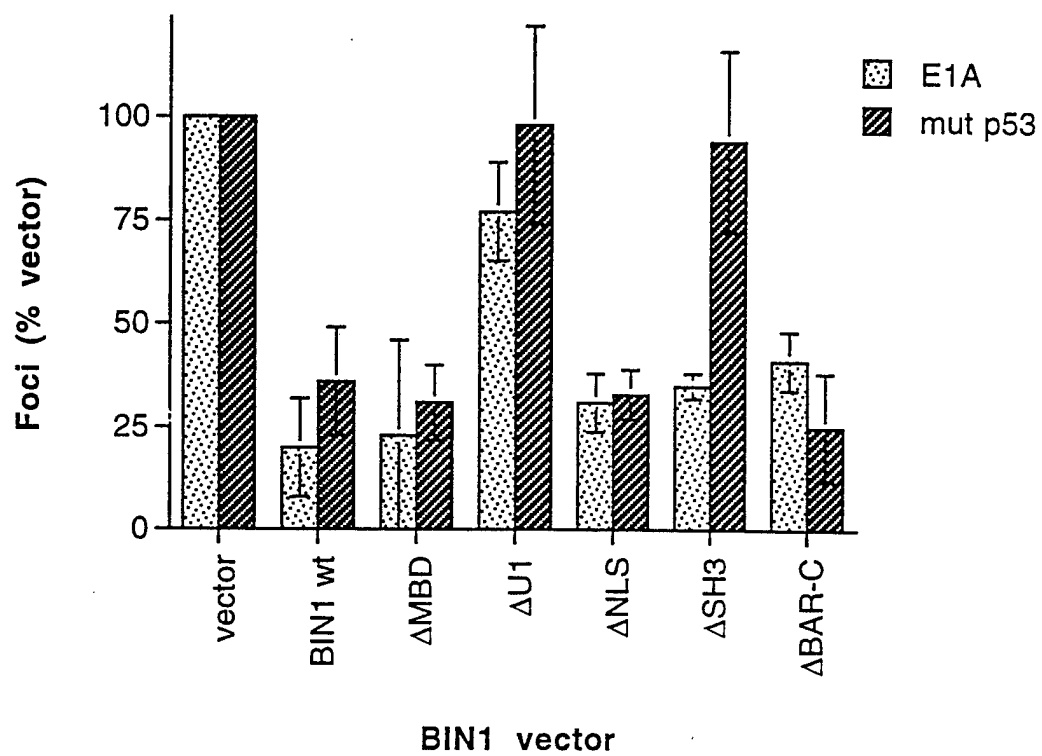


Fig. 6

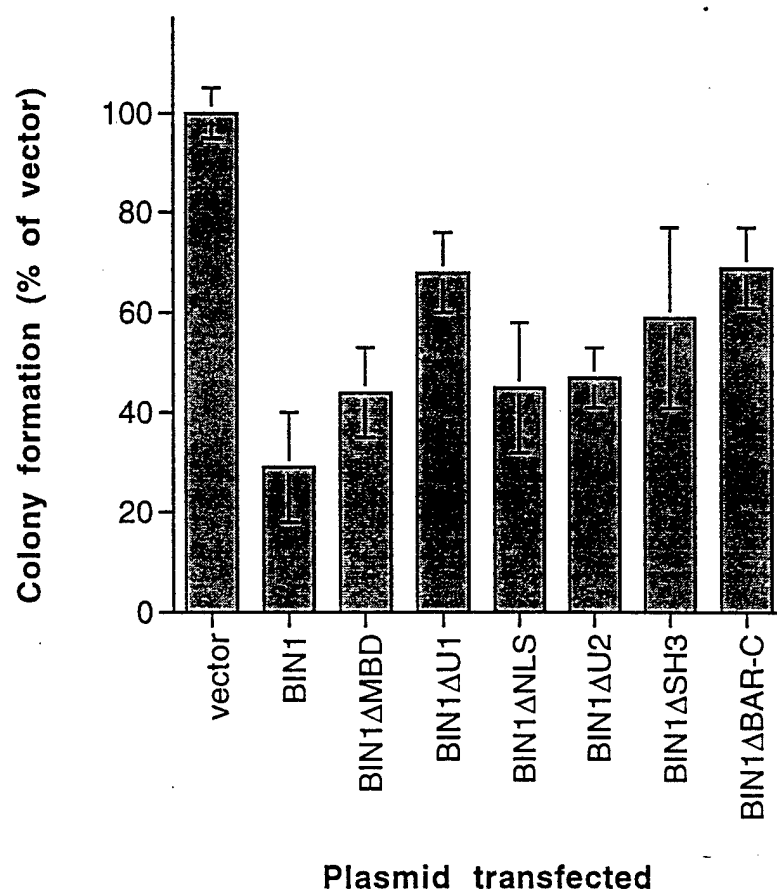


Fig. 7

